



The Generation of Novel Fluorinated Rapamycin Analogues

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Abstract

The aim of the project is to produce fluorinated rapamycin analogues using precursor directed biosynthesis in order to have a better understanding of the binding of rapamycin with FKBP12 and FRAP forming together a ternary complex responsible for rapamycin's immunosuppressant and anticancer activity. Fluorinated starter units must be synthesised and fed to *S. hygroscopicus* MG-210 a modified organism that does not produce the natural starter unit derived from the shikimic acid pathway. The strain *S. hygroscopicus* MG-210 was produced and provided by *Biotica Technology Ltd*.

3-Fluorocyclohexanecarboxylic acid, 4-fluorocyclohexanecarboxylic acid and 3-fluorocyclohexanecarboxylic acid were first synthesised and fed to *S. hygroscopicus* MG-210, but no incorporation was observed. Previous feedings carried out by *Biotica Technology Ltd* correlated to the non incorporation of the fluorinated starter units led to the conclusion that a fluorinated starter unit could only be incorporated if a hydroxyl group is present in position 3 or 4 of the fluorocyclohexanecarboxylic acid. A range of six fluorohydrins (1R*, 3S*, 4S*)-ethyl 4-fluoro-3-hydroxycyclohexanecarboxylate, (1R*, 3S*, 4S*)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate, (1R*, 3R*, 4R*)-ethyl 4-fluoro-3-hydroxycyclohexanecarboxylate, (1R*, 3R*, 4R*)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate, (1R*, 3R*, 4S*)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate and (1R*, 3S*, 4R*)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate were synthesised and fed to *S. hygroscopicus* MG-210 producing for the first time 6 different fluorinated prerapamycin analogues, with various production levels. The incorporation levels indicate that for optimum incorporation the hydroxyl group has to be in position 4 and that both ester and hydroxyl group must stand in the equatorial position. The best incorporated molecule was the all equatorial (1R*, 3R*, 4R*)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate. It was fed at a larger scale and the corresponding fluorinated prerapamycin analogue was isolated and its structure characterised using various 2D NMR techniques.

Publications from this work

1- Generating rapamycin analogues by directed biosynthesis: starter acid substrate specificity of mono-substituted cyclohexane carboxylic acids

R. J. M. Goss, S. E. Lanceron, N. J. Wise and S. J. Moss, *Org. Biomol. Chem.*, 2006, **4**, 4071-4073

2- An Expeditious Route to Fluorinated Rapamycin Analogues by Utilising Mutasynthesis

R. J. M. Goss, S. E. Lanceron, A. Deb Roy, S. Sprague, M. Nur-e-Alam, D. L. Hughes, B. Wilkinson, S. J. Moss, *ChemBioChem*, 2010, **11**, 698-702

Declaration

To the best of my knowledge this thesis presents my original work and research except when specifically referred to.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

TABLE OF CONTENTS

Acknowledgements	7
Abbreviation list.....	8
 I- INTRODUCTION CHAPTER.....	 11
1 INTRODUCTION	12
1.1 Pharmaceutical Drugs and Natural Products.	12
1.2 The History of Rapamycin	18
2 RAPAMYCIN'S SPECIFIC IMMUNOSUPPRESSIVE PATHWAY	19
2.1 Natural Products and Immunosuppression	19
2.2 Comparison between Rapamycin, FK506, Cyclosporin Immunosuppressive Pathways.	21
2.2.1 <i>T lymphocyte: the base of the immune response.</i>	21
2.2.2 <i>Differences and similarities in their proteomic targets and pathway.</i>	21
2.3 The Origin of Rapamycin's Immunosuppressive Activity: Structure and Activity of the Ternary Complex.....	24
2.3.1 <i>Crystal structure of the ternary complex compared to the binary complex.</i> ..	24
2.3.2 <i>Interactions between both proteins.</i>	28
2.3.3 <i>Chemical modification on rapamycin and consequences on its binding and activity.</i>	31
3 BIOSYNTHESIS OF RAPAMYCIN	34
3.1 Starter Unit of the Polyketide Synthesis	34
3.1.1 <i>The shikimic acid pathway</i>	34
3.1.2 <i>Route from shikimic acid to the determination of the true starter unit</i>	36
3.2 The Polyketide Biosynthesis.....	38
3.2.1 <i>Common elements between fatty acid biosynthesis and PKS Type 1, 2, 3</i>	38
3.2.2 <i>Rapamycin's PKS</i>	39
3.3 The Pipecolic Ring.....	44
3.4 The Assembling of the Macrocycle and the End of the Biosynthesis of Rapamycin..	45
4 AIMS OF THE PROJECT	50
 II- RESULTS AND DISCUSSION CHAPTER.....	 56
1 SYNTHESIS TOWARD MONOFLUORINATED AND DIFLUORINATED CYCLOHEXANECARBOXYLIC ACIDS AND 3-FLUOROCYCLOHEXENE- CARBOXYLIC ACID.	57
1.1 Synthetic Route Toward 4-Fluorocyclohexanecarboxylic Acid 10 and 4,4- Difluorocyclohexanecarboxylic Acid 14.	60
1.1.1 <i>The reduction step</i>	61
1.1.2 <i>The monofluorination step.</i>	63
1.1.3 <i>Knoevenagel type reaction converting the ketone group into a nitrile of the monofluorinated compound.</i>	65
1.1.4 <i>Hydrolysis of the nitrile into the acid.</i>	66
1.1.5 <i>Difluorination of the ketone</i>	67
1.1.6 <i>Knoevenagel type reaction on the difluorinated compound.</i>	68
1.1.7 <i>Hydolysis attempt of the nitile group in the acid of the difluorinated compound.</i>	69
1.2 Synthesis of 3-Fluoro-cyclohex-1-enecarboxylic Acid and Attempt to Reduce its Double Bond.	70
1.2.1 <i>Synthesis of 3-fluoro-cyclohex-1-enecarboxylic acid.</i>	70
1.2.2 <i>Double bond reduction attempt.</i>	71
1.3 3-Fluorocyclohexanecarboxylic Acid Synthesis.....	73

1.4 Attempt to synthesise 2-fluorocyclohexanecarboxylic acid.	74
2 SYNTHESIS TOWARDS FLUOROHYDRIN CYCLOHEXANOIC ESTERS.....	76
2.1 Synthesis towards 4-Hydroxy, 3-Fluorocyclohexanoic Methyl Ester.....	78
2.1.1 Synthesis towards (1 <i>R</i> *, 3 <i>S</i> *, 4 <i>S</i> *)-methyl 3-fluoro-4-hydroxycyclohexanecarboxylate.	78
2.1.2 Epimerisation attempt on the methyl ester.	81
2.2 Fluorohydrin Ethyl Ester Synthesis.....	82
2.2.1 Synthesis towards (1 <i>R</i> *, 3 <i>S</i> *, 4 <i>S</i> *)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate.	82
2.2.2 THP protection and optimisation of the separation between both isomers. ..	87
2.2 Epimerisation of the Ethyl Ester	89
2.2.1 Epimerisation kinetic conditions.....	90
2.2.2 Thermodynamic epimerisation using potassium <i>tert</i> -butoxide.	94
2.3 Alcohol Inversion	99
2.4 Confirmation of the Stereochemistry of Fluorohydrin cyclohexane Methyl and Ethyl Esters.....	102
2.5 Global Synthesis of 6 Different Ethyl Ester Cyclohexane Fluorohydrins.....	104
3 FEEDING OF THE MOLECULES TO <i>S. HYGROSCOPICUS</i> MG-210.....	105
3.1 Previous Feedings of 6 Carbon Membered Ring Starter Units.....	105
3.2 Feeding of the Hydroxycyclohexanecarboxylic Acids and Fluorinated Cyclohexanecarboxylic Acids.....	107
3.3 Feeding of the 6 fluorohydrins.....	110
3.4 Scale up Feeding.....	112
CONCLUSION	120
III- EXPERIMENTAL SECTION CHAPTER.....	126
1 SYNTHESIS TOWARDS MONOFLUOROCYCLOHEXANECARBOXYLIC ACIDS 4,4-DIFLUOROCYCLOHEXANECARBOXYLIC ACID AND 3-FLUOROCYCLOHEXENECARBOXYLIC ACID.	128
1.1 Synthesis towards 4-Fluorocyclohexanecarboxylic Acid.....	128
1.2 Synthesis towards 4,4-Difluorocyclohexanecarboxylic Acid.....	130
1.3 Synthesis towards 3-Fluorocyclohexenecarboxylic Acid.....	132
1.4 Synthesis toward 3-Fluorocyclohexanecarboxylic Acid.....	134
1.5 Synthesis toward 2-Fluorocyclohexanecarboxylic Acid.....	140
2 SYNTHESIS OF THE FLUOROHYDRIN CYCLOHEXANECARBOXYLIC METHYL AND ETHYL ESTERS.....	141
2.1 Synthesis towards 3-Hydroxy-4-fluorocyclohexane carboxylic Methyl Ester.	141
2.2 Synthesis towards Fluorohydrincyclohexanecarboxylic Ethyl Esters and Derivatives.	144
3 ADMINISTERING THE SHIKIMIC ACID ANALOGUES TO <i>S. HYGROSCOPICUS</i> MG-210 CULTURES	163
3.1 Small Scale Feeding of Shikimic Acid Analogues	166
3.2 Large scale feeding of the all equatorial fluorohydrin to <i>S. hygrosopicus</i> MG-210.	168
REFERENCES.....	172
APPENDIX 1: ¹ H NMR Spectrum of Fluoropreraamycin	176
APPENDIX 2: ¹³ C NMR Spectrum of Fluoropreraamycin	177
APPENDIX 3: DEPT NMR Spectrum of Fluoropreraamycin	178
APPENDIX 4: COSY NMR Spectrum of Fluoropreraamycin.....	179
APPENDIX 5: HSQC NMR Spectrum of Fluoropreraamycin.....	180
APPENDIX 6: HMBC NMR Spectrum of Fluoropreraamycin.....	181

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Abbreviation list

A°: Angstrom
ACP: acyl carrier protein
AT: acyl transferase
Alloc: allyloxycarbonyl
Asp: aspartic acid
Boc: N-*tert*-butoxycarbonyl
bs: broad singulet
CoL: CoA ligase
EtOH: ethanol
d: doublet
DAHP: 2-dehydro-3-deoxyphosphoheptonate aldolase
DAST: diethyl amino sulphur trifluoride
DCC: dicyclohexylcarbodiimide
DCM: dichloromethane
6-dEB: 6-deoxyerythronolide B
DEBS: deoxyerythronolide B synthase enzyme
DH: dehydratase
DMAP: 4-dimethylaminopyridine
DME: 1,1-dimethoxyethane
DHP: 3,4-dihydro-2H-pyran
DMF: N,N-dimethylformamide
DMP: Dess–Martin periodinane
DMSO: dimethyl sulfoxide
DNA: Deoxyribonucleic acid
ER: enoyl reductase
ESI: electrospray ionization
Et: ethyl
EtOH: ethanol
FRAP: FK506 and rapamycin associated protein
FRB: FKBP12-Rapamycin Binding domain
FK506: Tacrolimus
FK520: Ascomycin
FKBP: FK506 binding protein

g: gramme
G: gap
Gln: glutamine
Glu: glutamic acid
GM: genetically modified
IL-: interleukin receptor
Ile: isoleucine
IP₃: Inositol trisphosphate
kD: kilo Dalton
KR: ketoreductase
KS: ketosynthase
L: liter
Leu: leucine
LiHMDS: Lithium bis(trimethylsilyl)amide
Lys: lysine
M: mitosis
m: multiplet
Me: methyl
MeOH: methanol
MHC: major histocompatibility complex
min.: minute
Ms: mesyl
mTor: mammalian target of rapamycin
NCI: National Cancer Institute
NMR: nuclear magnetic resonance
NRPS: non ribosomal peptide synthetase
OMs: mesylate
p-: para
Phe: Phenylalanine
PKS: polyketide synthase
Py : Pyridine
PNB: Para Nitrobenzoyl
s: singlet
S.: *Streptomices*
Ser: serine

SPR: surface plasmon resonance

TBDPS: *tert*-butyldiphenylsilyl

TE: thioesterase

TES: triethylsilyl

THC: tetrahydrocannabinol

THF: tetrahydrofuran

THP: tetrahydropyran

Thr: threonine

TIPS: triisopropylsilyl

TLC: thin layer chromatography

TosMic: tosyl methyl isocyanate

Trp: tryptophan

Ts: tosyl

Tyr: tyrosine

Val: valine

I- INTRODUCTION CHAPTER

1 INTRODUCTION

1.1 Pharmaceutical Drugs and Natural Products.

Pharmaceutically active natural products have been in use since ancient times to prevent or cure illnesses. They have also found use as poisons and for recreational purposes. One early example was the use of curare, extracted from plants of the Amazon (by the indigenous South Americans), as a poison with which to coat arrow tips.¹ In controlled doses, the neuromuscular inhibitory property of this compound and its derivatives has found a modern use as an anaesthetic. Around two hundred years ago, Friedrich Sertürner, a young pharmacist, isolated morphine **1.1**.² It was the first time a natural product was isolated. Morphine **1.1** could therefore be precisely dosed for pharmaceutical usage as an analgesic to relieve severe pains. Historically the majority of patented pharmaceutical drugs are derived from natural products.² Though many of these natural products and derivatives are prepared synthetically, a few commercially important natural product derived drugs are still extracted from their original natural source. One such example is morphine **1.1** produced by opium poppies *Papaver somniferum*,² or tetrahydrocannabinol **1.2** (THC) from the cannabis plant *Cannabis sativa*. Other medicinally important natural products are produced using enhanced biological techniques in order to improve the yields, for example the industrial generation of plant cell cultures in order to produce taxol **1.3**² (it was originally extracted from the bark of the European yew *Taxus brevifolia*, but it was not a sustainable source²) or deep-tank fermentation for mass production of penicillin G **1.4**, erythromycin **1.5** and other antibiotics (**Figure 1.1**).

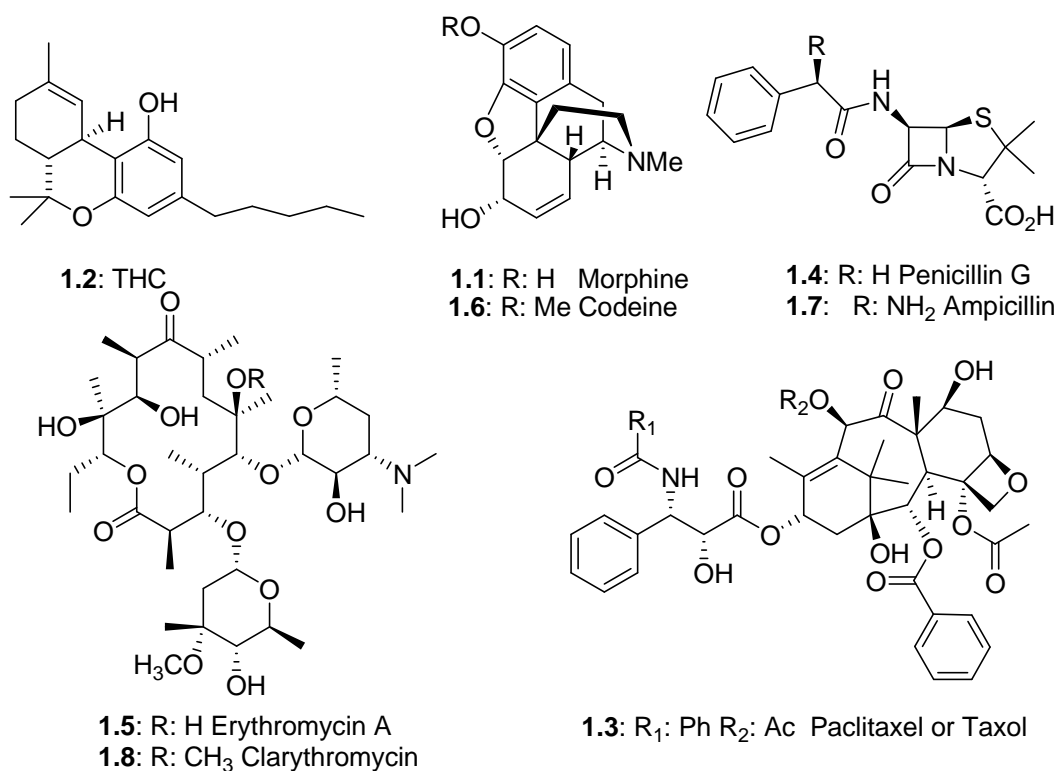
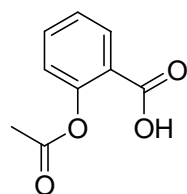


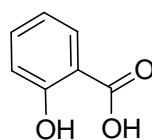
Figure 1.1: Example of unmodified natural products and semi synthetic derivatives.

A large number of drugs are semi synthetic derivatives of the natural metabolites, such as codeine **1.6**, ampicillin **1.7** or clarythromycin **1.8** (see **Figure 1.1**). Originally isolated from opium poppies like morphine **1.1** but in much smaller quantities, codeine **1.6** is produced industrially by phenolic *O*-methylation of morphine **1.1**.³ Clarythromycin **1.8** is produced by *O*-methylation of erythromycin A **1.5**. Ampicillin **1.7** is an aminated derivative of penicillin G **1.4**. An alternative to the traditional chemical semi synthetic route is an efficient two-step, one-pot enzymatic synthesis of ampicillin **1.7** from penicillin G **1.4** recently reported by Lin and coworkers using a Penicillin acylase in the presence of *D*-phenylglycine methyl ester in the presence of organic solvents and water.⁴

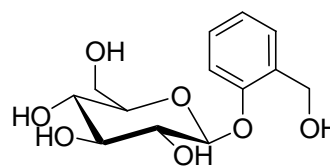
Parts of the pure synthetic drugs mimic the structure of natural products, such as aspirin **1.9**. Salicylic acid **1.10**, the active form is also obtained as a metabolite of salicin **1.11** present in willow bark (used by ancient civilisations as a pain killer **Figure 1.2**).¹



Aspirin
1.9



Salicylic acid
1.10

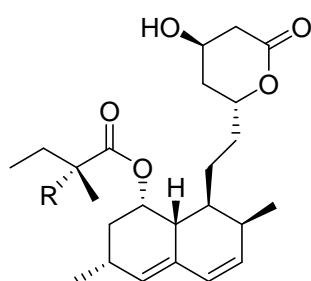


Salicin
1.11

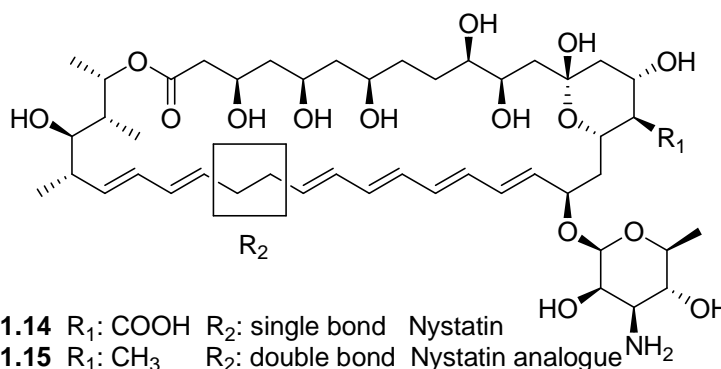
Figure 1.2: Chemically synthesised aspirin and natural product salicin have salicylic acid as their common active metabolite.

More recently, some natural products have also been produced through biosynthetic engineering. This often involves introducing a gene sequence from the wild type organism coding for the synthesis of the metabolite in a host organism. Human insulin is mainly synthesised in modified *Escherichia coli* by pharmaceutical companies, the traditional alternative being extracting similar insulin exclusively from animal pancreas.⁵

Biosynthetic engineering has also been used more recently on molecules, for which biosynthetic genes have been sequenced to generate new designer natural product analogues. This involves genetically modifying the sequence responsible for the synthesis of the metabolite introducing, replacing, or deleting genes to obtain a modified metabolite. Most of these compounds originate from polyketide biosynthesis and NRPS. Simvastatin **1.12**, an analogue of Lovastatin **1.13** was produced using an engineered biosynthetic approach by Tang and co-workers using a modified strain of *Aspergillus terreus*⁶ (produced traditionally in a semi synthetic manner).² The Nystatin analogue **1.15** also obtained in this manner is currently in clinical trials and is showing an improved antifungal activity and lower toxicity compared to the parent compound **1.14** (Figure 1.3).



1.12 R: CH₃ Simvastatin
1.13 R: H Lovastatin



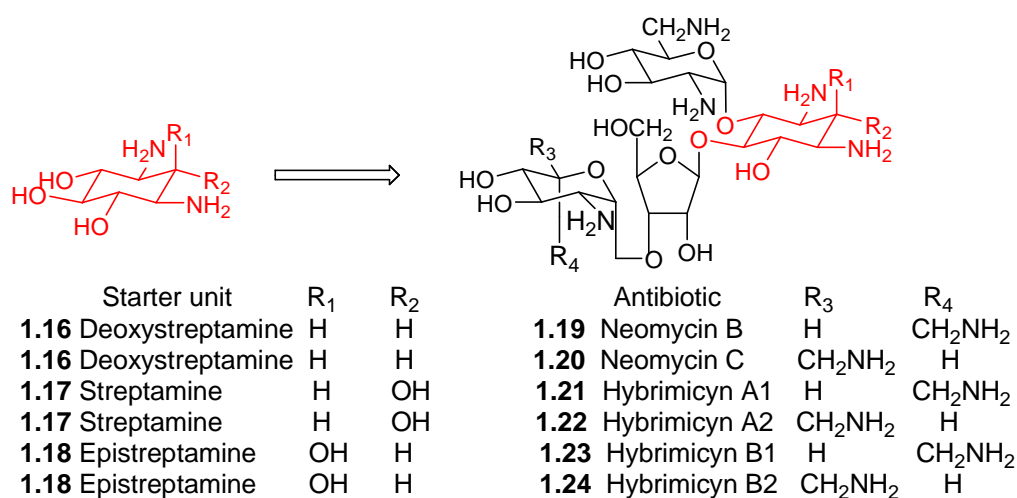
1.14 R₁: COOH R₂: single bond Nystatin
1.15 R₁: CH₃ R₂: double bond Nystatin analogue

Figure 1.3: Example of analogues obtained biosynthetically by a bioengineered organism.

Although very promising, producing new analogues, by using combinatorial biosynthesis is very time consuming and complicated, making the new analogues fairly expensive to produce.

An alternative approach which combines chemistry and biosynthesis is precursor directed biosynthesis. This usually consists of feeding an exogenous alternative starter unit to the natural producing microorganism to obtain, if incorporated, a mixture of the natural metabolite and the new analogue. Once the flexibility of the starter unit is demonstrated, the yields can be improved by bioengineering a modified microorganism, inhibiting the production of the natural precursor which previously is in competition with the exogenous one. The alternative starter unit can then be fed to the modified microorganism, obtaining the new analogue in a higher yield. Some substrates previously not significantly incorporated by the wild type organism, can be incorporated by the mutant because of the lack of competition with the natural starter unit.

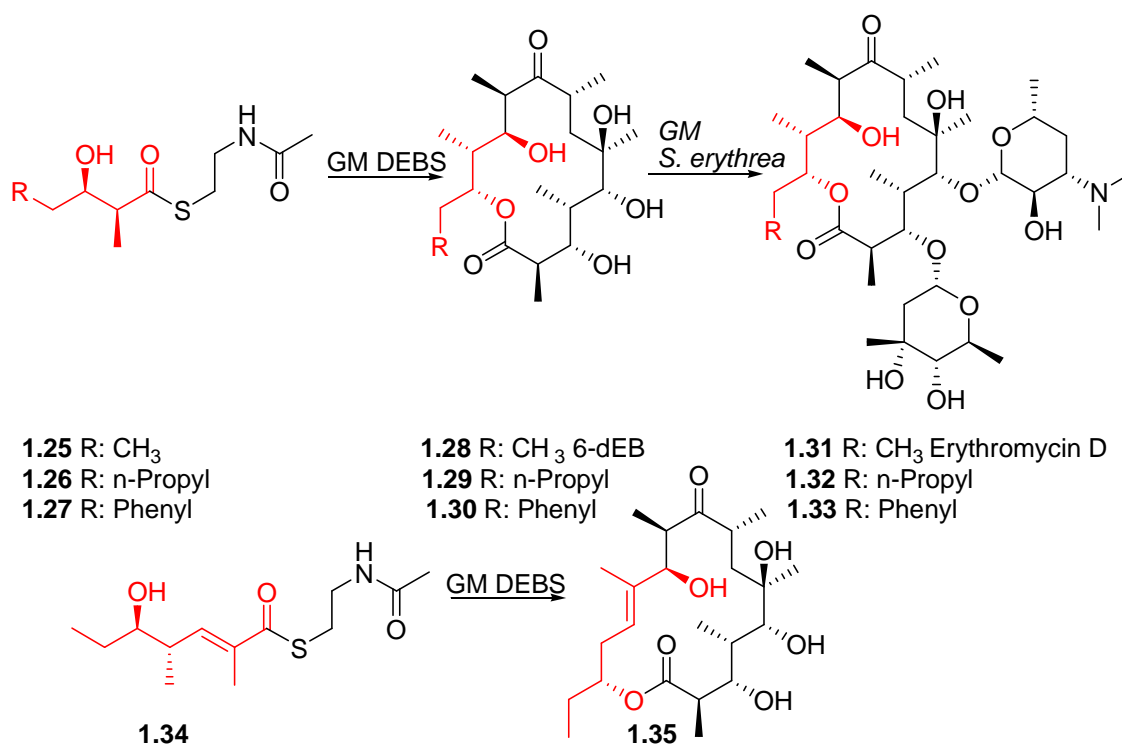
This procedure entitled ‘mutasynthesis’ was first reported in 1969.⁷ Neomycin B **1.19** and C **1.20** analogues naturally produced by *Streptomyces fradiae* were produced in this manner by Shier and coworkers (**Scheme 1.1**).⁷ A mutant strain of *Streptomyces fradiae*, in which the production of the natural precursor deoxystreptamine was abolished, successfully incorporated endogenous deoxystreptamine **1.16**, streptamine **1.17** and epistreptamine **1.18**. This resulted in the production of the expected neomycin B **1.19** and C **1.20** when deoxystreptamine **1.16** was fed, but also hydroxylated analogues **1.20** and **1.21** when streptamine **1.17** was fed, and diastereomeric hydroxylated analogues **1.23** and **1.24** when epistreptamine **1.18** was fed. The mutant strain at the time had been obtained by a random mutation caused by chemical mutagenesis through the use of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (**Scheme 1.1**).



Scheme 1.1: First precursor directed biosynthesis.

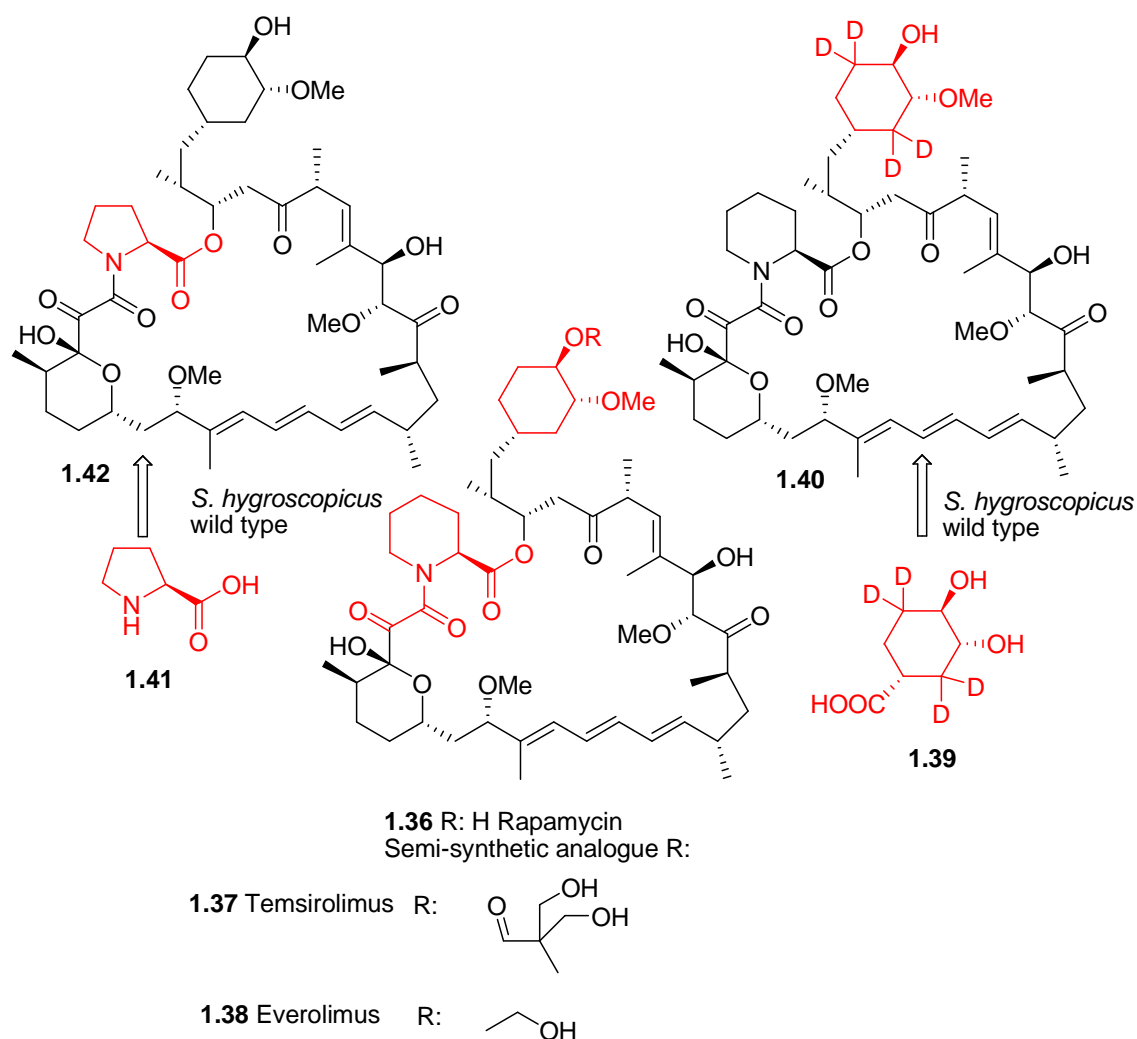
At this time mutant organisms were obtained usually with mutagenic chemicals or radiation by random screening. Until the 1990s precursor directed biosynthesis combined with mutagenesis was not a common method, as the mutations occurred in a random manner. More recently the progress of gene sequencing technology means that specific mutations can be made based on known sequences, therefore the method of mutasynthesis is more frequently used. The genes responsible for production of a precursor can be easily localised and inhibited, and the range of synthetic molecules and methods to produce them have advanced.

Erythromycin D **1.31** and analogues **1.32**, **1.33** were obtained and reported by John R. Jacobsen and co-workers (**Scheme 1.2**) in 1997 using mutasynthesis.⁸ The exogenous precursors **1.25**, **1.26**, **1.27**, **1.34** were fed to a modified mutant of the deoxyerythronolide B synthase enzyme (DEBS) with an inhibited production of the natural precursor *N*-acetyl cysteamine. Naturally DEBS produces 6-dEB **1.28** an intermediate to erythromycin D. Exogenous *N*-acetyl cysteamine **1.25** was incorporated producing 6-dEB **1.28**. Three other alternative starter units **1.26**, **1.27**, **1.34** gave three new analogues of 6-dEB **1.29**, **1.30** and **1.35** one of which **1.35** had an extended lactone ring from fourteen to a sixteen membered one (**Scheme 1.2**). The two fourteen-membered lactone analogues **1.29**, **1.30** and 6-dEB **1.28** produced by the modified DEBS were then fed to an engineered strain of *Saccharopolyspora erythrea* (natural producer organism of Erythromycin D) in which the production of 6-dEB was inhibited. This enabled the glycolisation of the macrocycle, erythromycin D **1.31** and two new analogues **1.32** and **1.33** were obtained in this manner (**Scheme 1.2**).



Scheme 1.2: First reported erythromycin analogues produced by precursor directed biosynthesis.⁸

Rapamycin **1.36** (Scheme 1.3), like erythromycin, is a biologically active natural product of polyketide origin. It is currently used as an immunosuppressive for kidney transplant patients. Two semi-synthetic derivatives Temsirolimus **1.37** and Everolimus **1.38** with a modified cyclohexyl moiety have recently been accepted as an anticancer treatment (Scheme 1.3). Precursor directed biosynthesis produced analogues of rapamycin with a modified cyclohexyl ring were first reported by Leadlay, Staunton and coworkers in 1996.⁹ Exogenous deuterated synthetic putative starter units **1.39** was fed to the natural producing microorganism *S. hygroscopicus* which led to the production of deuterated rapamycin analogue **1.40**, in parallel with normal rapamycin **1.36** (Scheme 1.3). Precursor directed biosynthesis was also used to make pipicolinate analogues of rapamycin by Leadlay and coworkers in 1998.¹⁰ Proline **1.41** was fed to wild type *S. hygroscopicus* and an engineered variant with reduced production of the pipicolinyl acid. In both cases the proline rapamycin analogue **1.42** was produced (Scheme 1.3).¹⁰ Wild type *S. hygroscopicus* demonstrates a level of substrate flexibility. Modified strains of *S. hygroscopicus* (generated by *Biotica Technology Ltd*), in which production of the starter unit responsible for the formation of the cyclohexyl moiety is inhibited, have been used for this research. Exogenous starter units can then be fed to the modified *S. hygroscopicus* in order to create a bigger range of rapamycin analogues. The activity of the new analogues can then be investigated.



Scheme 1.3: Rapamycin, two semi-synthetic rapamycin analogues currently used for cancer treatment and two different examples of precursor directed biosynthetic analogues obtained with the wild type microorganism.

In addition to its anticancer and immunosuppressive properties, rapamycin shows promising results for treatment of a whole range of conditions from autism to tuberous sclerosis. It was recently reported in *Nature* that rapamycin could extend the life of mice.¹¹ Rapamycin has great therapeutic potential, but it was only due to the perseverance of Sehgal, the researcher who discovered rapamycin, that it was ever developed.

1.2 The History of Rapamycin

Rapamycin is a secondary metabolite produced by *Streptomyces hygroscopicus* an actinomycete originally discovered in a soil sample of Rapa-Nui (the native name of the Easter Island).^{12, 13} It was first isolated in 1972 by Ayerst Research Laboratories in

Montreal, and its structure was published in 1975.¹² Rapamycin is a 31-membered macrolactone polyketide featuring a cyclohexyl moiety derived from the shikimic acid pathway, a pipercolinyl ring and a triene in its structure. It was first developed for its antifungal properties,¹⁴ but was abandoned when it was shown, that it caused atrophy of the thymus and the spleen.¹⁵ Although it was dropped as a potent antifungal, it was found to have immunosuppressive and antiproliferative activity but this was not exploited at the time. Dr Suren Sehgal who was part of the Ayerst team of scientists that isolated rapamycin, believed it could have anticancer properties and sent a sample to the National Cancer Institute (NCI). The NCI found that it had a remarkable activity against solid tumours and it was placed on their priority list. It was the first potential anticancer agent with cytostatic and not cytotoxic activity. After difficulties by Ayerst to develop an intravenous formulation for clinical trials the rapamycin project was dropped after financial restructuring of the company. In 1987, Ayerst merged with Wyeth and changed management. This, combined with the fact that FK506, structurally related to rapamycin, was identified as a potential immunosuppressive drug, led to rapamycin being tested on animals under the direction of Dr Suren Sehgal. The results convinced Wyeth-Ayerst to redevelop rapamycin as an immunosuppressant, and continue investigating its antitumor and antiproliferative properties. In 1999 it was approved as an anti-rejection drug under the name Rapamune. It is particularly used for kidney transplants as it presents very little nephrotoxicity compared to cyclosporin A and FK506, which were generally used previously.

Moreover after a range of semi-synthetic rapamycin analogues were tested as immunosuppressives and potential anticancer agents, two compounds were approved after clinical trials as anticancer drugs, Temsiromilus **1.37** in 2007, sold as Torisel by Wyeth and Everolimus **1.38** in 2009 sold as Afinitor by Novartis (**Scheme 1.3**). They are both used more specifically against kidney cancer. Everolimus had previously been approved as an immunosuppressive for kidney transplants under the name Certican.

2 RAPAMYCIN'S SPECIFIC IMMUNOSUPPRESSIVE PATHWAY

2.1 Natural Products and Immunosuppression

The natural product cyclosporin A **1.43** produced by the fungus *Beauveria nivea* first isolated from a soil sample in Norway revolutionised transplantation (**Figure 1.4**). It

allowed successful heart and liver transplantation and turned kidney transplantation into a fairly common procedure in spite of its nephrotoxic properties.¹⁵ Previously a mixture of azathioprine and steroids were used for kidney transplants, but only around 50% of people would live more than a year. Other transplantations such as heart or liver were experimental and would always lead to rejection of the organ and death of the subject. Later FK506 **1.44** also known as Fujimycin produced by *Streptomyces tsukubaensis* was isolated from a soil sample in Japan (**Figure 1.4**).¹⁵ This was found to have similar activity to cyclosporin in heart and renal transplants and even had enhanced results in liver transplant at a lower dosage.¹⁵ An ethyl analogue of FK506 named FK520 **1.45** also showed immunosuppressive activity. It was produced by *S. hygroscopicus* like rapamycin. FK520 is mainly used for treating dermatitis. Rapamycin **1.36**, FK506 **1.44** and FK520 **1.45** are structurally related (**Figure 1.4**).

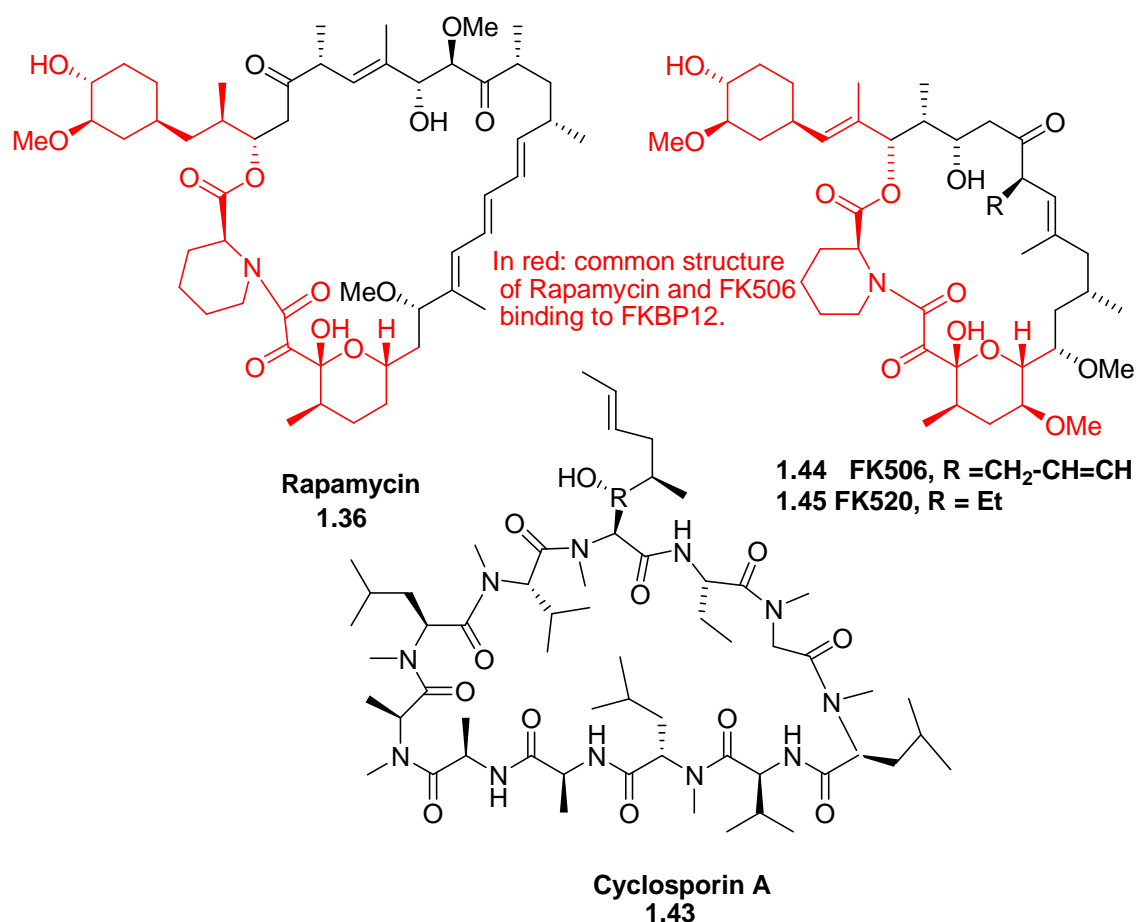


Figure 1.4: Main natural products with immunosuppressive activity.

2.2 Comparison between Rapamycin, FK506, Cyclosporin Immunosuppressive Pathways.

2.2.1 T lymphocyte: the base of the immune response.

The T lymphocyte is the most important cell involved in an immunological response. Most research on immunosuppressive compounds is directed toward establishing the mechanism of inhibition of the T cell.

In its early stages the T cell travels in the thymus and the glycoprotein antigen receptor present on its surface either reacts with a self-antigen and will receive an apoptotic signal resulting in cell death (negative selection) or it develops and interacts with a foreign antigen presented by MHC (major histocompatibility complex) and will receive a survival signal (positive selection). The cell will then migrate to the peripheral lymph nodes and will be able to initiate an immune response when its receptor associates with a foreign antigen presented by MHC. The immune response involves a cascade of intracellular signalling events leading to an activation and proliferation of T- cells and other cells also involved in the process and the production of a variety of effector molecules like interleukin-2 (IL-2).¹⁶ The signalling pathway of the immune response is not yet fully known in intimate detail.

2.2.2 Differences and similarities in their proteomic targets and pathway.

A) The proteomic targets

Due to their common structure it was first believed that the immunosuppressive mode of action of rapamycin and FK506 were similar. Moreover rapamycin and FK506 antagonise each others action probably through competitive binding to a common receptor. Early crystal structures of binary complexes¹⁷ confirmed that rapamycin shares the same cellular target as FK506, an immunophilin (peptidyl-prolyl isomerase) called FKBP12 (FK506 binding protein) due to their common structure (**Figure 1.4**) whereas cyclosporin A binds to a different type of immunophilin called cyclophilin.¹⁸ FKBP12 and cyclophilin are two different enzymes, both catalyzing the isomerisation of *cis* and *trans* amide bonds of peptide and protein substrates.¹⁹ Both rapamycin and FK506 inhibit FKBP12's peptidyl-prolyl isomerase activity. Although FKBP12 is inhibited by both immunosuppressants, FKBP12 was found not be directly implicated in the immune response. Both molecules were found to inhibit different pathways. Although FK506

and cyclosporin A bind to a different immunophilin their mode of action is identical, they both inhibit calcineurin, whereas rapamycin inhibits FRAP, also named mTOR (mammalian target of rapamycin).¹⁸ FRAP is a serine/threonine protein kinase that regulates cell growth, cell proliferation, protein synthesis and transcription.

Cyclosporin A binds with cyclophilin and calcineurin and FK506 binds with FKBP12 and calcineurin: the formation of both ternary complexes results in the inhibition of calcineurin. Calcineurin intervenes in the early stage of the immune response interfering with a calcium dependant signalling pathway. By inhibiting calcineurin the T cell cycle is stopped from G₀ (resting phase of the cell **Figure 1.5**) to G₁ (growth of the cell synthesising all the enzymes necessary for DNA synthesis **Figure 1.5**).

Rapamycin binds with FKBP12 and FRAP. The formation of this ternary complex results in the inhibition of FRAP. FRAP intervenes in the later stage of the immune response interfering with a calcium independent signalling pathway. As a result of the inhibition of FRAP, the cell cycle is stopped from G₁ to S (DNA replication **Figure 1.5**).

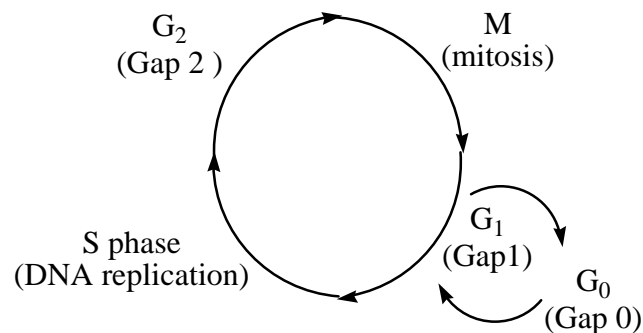


Figure 1.5: Cell cycle diagram

B) The immune response and its inhibition by cyclosporin A, FK506 and rapamycin.

The binding of the antigen with the T cell receptor leads to the activation of different kinases that activate various enzymes by phosphorylating them. This results later, in the production of IP₃ (Inositol trisphosphate). IP₃ is responsible for the opening of Ca²⁺ channels. This leads to an increase in the cytoplasmic level of calcium, which activates calcineurin.¹⁶ FK506 and cyclosporin disrupt the activation of calcineurin.

Activated calcineurin will normally activate NFAT (nuclear factor of activated T cells) by dephosphorylating it.¹⁶ NFAT is a transcription factor regulating interleukin 2 (IL-2). Once dephosphorylated, NFAT translocates into the T cell nucleus by diffusion and associates with other transcription factors from the AP1 family in order to transcript

interleukin-2 (IL-2). IL2 once produced will bind to IL-2 receptors on various cells including T-lymphocytes. Once IL-2 binds to its growth factor receptor, a multiprotein complex called CmTOR1 containing FRAP (also called mTor) is activated. If Rapamycin-FKBP12 is present it will disrupt the activation of the FRAP complex, by binding to FRAP.

The FRAP complex (CTOR1), once activated, can phosphorylate the p70 ribosomal protein S6 kinase ($P70^{S6K}$) on threonine 389 within its hydrophobic motif.^{20, 21} This phosphorylation is necessary for its activation. Once activated $P70^{S6K}$ will phosphorylate a serine residue on the 40S ribosomal subunit S6 protein responsible for translating certain m RNA's encoding for ribosomal proteins and elongation factors which are essential for the cell cycle to progress from G1 to S (**Figure 1.6**).²²

When rapamycin is present this pathway is inhibited and results in the non proliferation of the T cells and therefore immunosuppression.

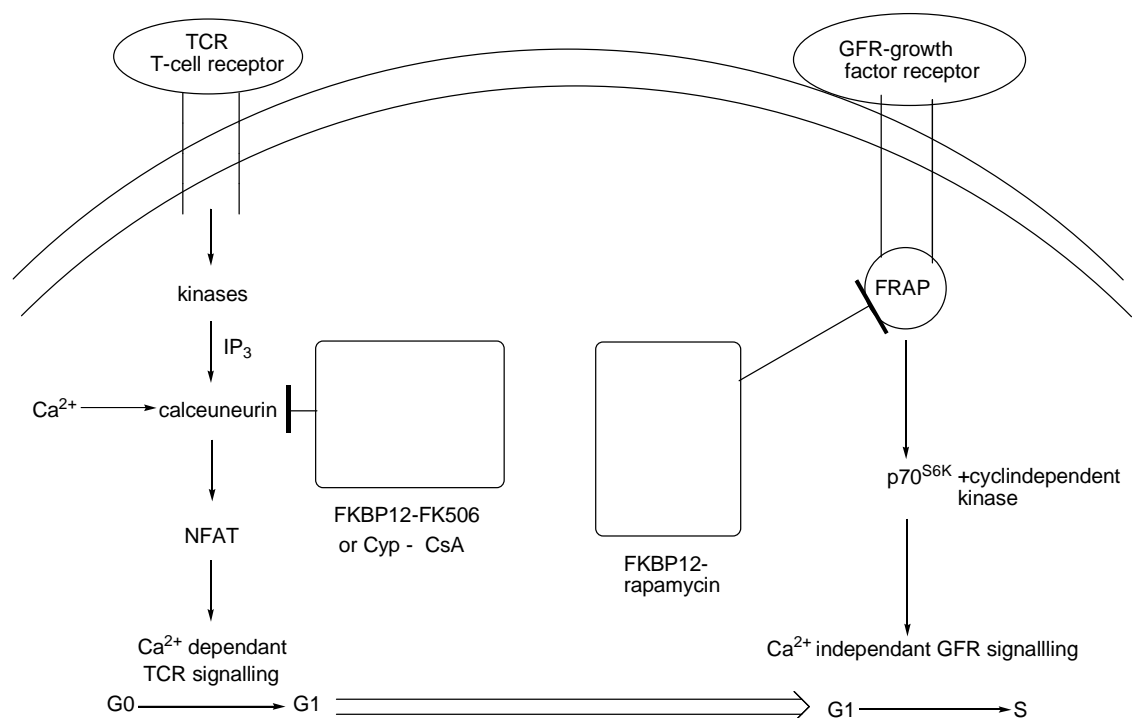


Figure 1.6: Pathways inhibited by rapamycin, cyclosporin and FK506.¹⁶

Calcineurin is usually specific to T cells whereas FRAP is a pivotal regulator of cell growth and proliferation of different types of cells including T cells.²³ It is present in many cells from yeast to humans and binds to growth factors such as IL-2 receptors as previously seen, IL-3 receptors, platelet-derived growth factor, epidermal growth factor, and insulin receptors. Therefore this confers rapamycin not only immunosuppressive activity but also antifungal and antitumor activities as it blocks the proliferation of various cells. Those cells can be tumour cells or healthy cells, T cells as seen previously

or fungal cells. Moreover in cancer cells the mTor signalling pathway is stimulated, with in certain cases like breast cancer, an over expression of p70^{S6K}.²⁴ More recently this pathway was shown to be involved in aging. Rapamycin extended the life expectancy of mice living in aseptic conditions.^{11, 25}

The activity of rapamycin is linked to its capacity to inhibit FRAP, by binding with it and with FKBP12. FKBP12 or rapamycin on their own do not inhibit FRAP. The capacity of rapamycin to form this ternary complex with FRAP and FKBP12 is essential for its immunosuppressive activity.

2.3 The Origin of Rapamycin's Immunosuppressive Activity: Structure and Activity of the Ternary Complex.

2.3.1 Crystal structure of the ternary complex compared to the binary complex.

Before rapamycin and the 12 kDa protein FKBP12 bind to FRAP to form a ternary complex they first bind to each other. Choi and coworkers observed from a crystal structure of the FKBP12-rapamycin binary complex that rapamycin binds to a hydrophobic pocket of FKBP12, leaving about 50% of its surface exposed on the exterior of the complex.²⁶

The FKBP12-rapamycin complex binds to FRAP through a 100 amino acid domain²⁷ called FRB that can be expressed as a 12 kDa soluble protein.²⁶ The entire FRAP protein is 289 kDa.

Once the FKBP12-rapamycin-FRB ternary complex was obtained, Choi solved the crystal structure using a combination of molecular isomorphous replacements.²⁶ The crystal structure showed that FRB and FKBP12 bind together with rapamycin occupying two different hydrophobic binding pockets simultaneously, one in the FRB domain and the other in FKBP12. Both binding pockets are composed of aromatic residues. The structure showed extensive interactions between rapamycin and both proteins in both binding pockets, but fewer between the proteins themselves.

The ternary complex was described by Choi as a roughly rectangular shape with overall dimensions of 60 Å, 45 Å and 35 Å. The size of FRB and FKBP12 is roughly equivalent and rapamycin is almost completely buried between both proteins.

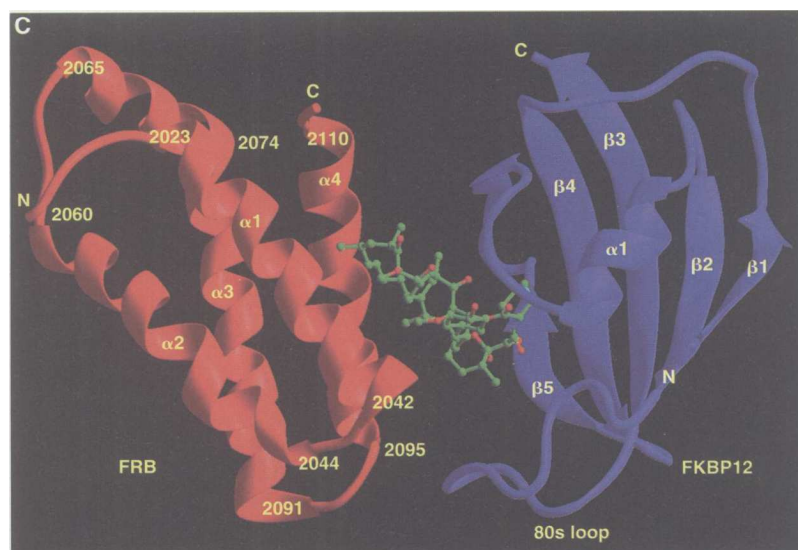


Figure 1.7: Structure of the ternary complex resolved by Choi and coworkers showing in red the FRB domain, in blue the FKBP12 and in green rapamycin.²⁶

FKBP12 contains a large β sheet composed of five anti-parallel β strands pressed against a short amphipathic α helix. Together the β sheet and α helix form the hydrophobic pocket in which rapamycin binds. Three loops surround and contribute to the binding pocket; the 40s loop described as a bulge in $\beta 5$,²⁶ the 50s loop linking $\beta 5$ to α , and the 80s loop connecting $\beta 2$ to $\beta 3$ (**Figure 1.7**).²⁶ Choi described the structure of FKBP12-rapamycin in ternary complex as very similar to the one of the binary complex without rapamycin.

Choi described the structure of the FRB domain of FRAP as a bundle of four helices linked to each other through short underhand connections. All four helices (ignoring the first 10 residues of $\alpha 3$) are about 26 Å long, each helix containing between 16 to 19 residues. The $\alpha 1$ and $\alpha 2$ helices seem almost parallel (interhelical angle of 22°), and so do $\alpha 3$ and $\alpha 4$ (20°). The crossing angle between $\alpha 1$, $\alpha 2$ and $\alpha 3$, $\alpha 4$ ranges from 30° to 60°.²⁶ The parallel helices are closer to each other and show the shortest interhelical distances. Most of the hydrophobic and aromatic residues are found in the interhelical regions, and the hydrophilic residues are exposed to solvent. Choi determined that the crossing of $\alpha 1$ and $\alpha 4$ formed close to its crossing point a deep crevice lined by six aromatic side chains constituting the hydrophobic pocket in which rapamycin binds to FRB.²⁶

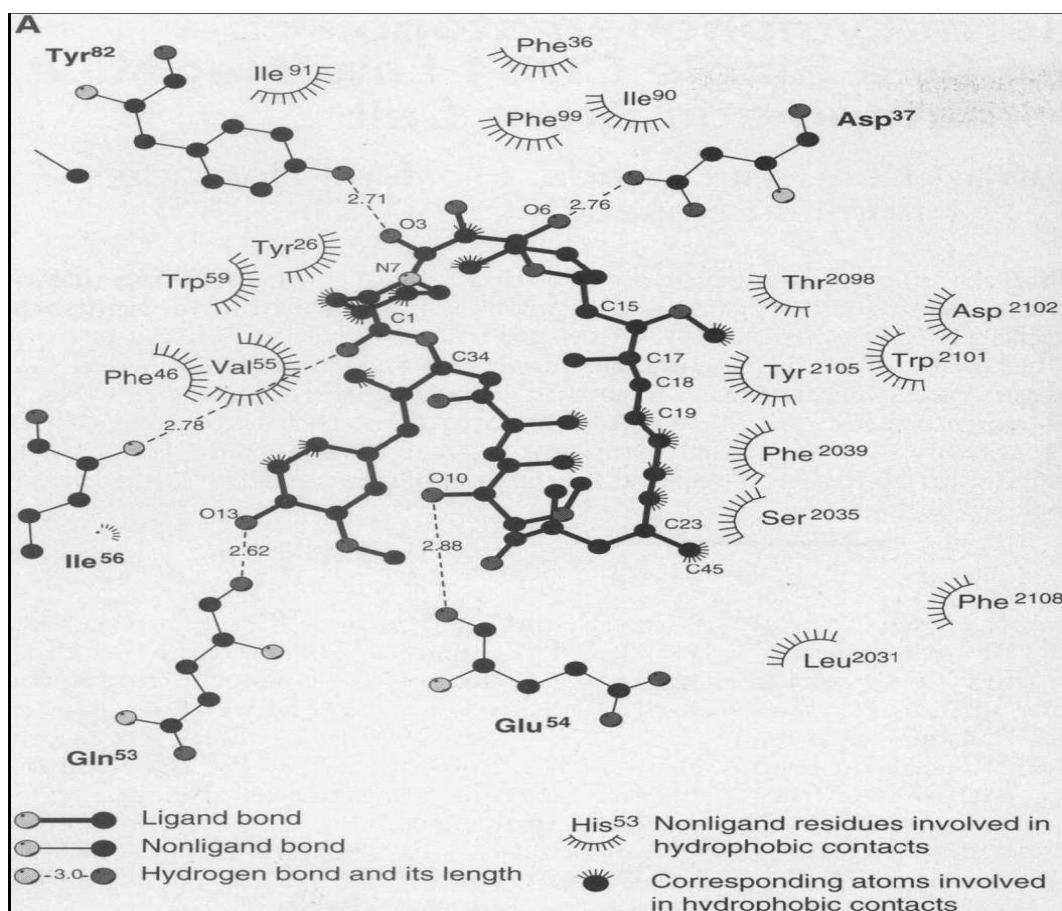


Figure 1.8: Structure from Choi and coworkers showing the interactions of rapamycin with FKBP12 and FRB.²⁶

Rapamycin's interactions with FKBP12 in the ternary complex are very similar to those of the binary complex. The crystal structure showed that rapamycin has extensive hydrophobic contacts with aromatic residues of FKBP12, and establishes five hydrogen bonds (**Figure 1.8**) with FKBP12. Trp59 forms the base of the binding pocket and is in contact with the most buried portion of rapamycin the pipercolinyl ring. C3, C4 and C5 of the pipercolinyl ring are involved in hydrophobic contacts with FKBP12. Other hydrophobic contacts with FKBP12 involve the methyl groups on C29, C31, C35 and C11. C41 and C42 of the cyclohexyl moiety are also involved in hydrophobic contacts with FKBP12 and so is C9. Three of the five hydrogen bonds involve hydroxyl groups on C10, C28 and the C40 of the cyclohexyl moiety. The two final hydrogen bonds concern the double bonded oxygens on C1 and C8 (**Figure 1.8** and **1.9**).²⁶

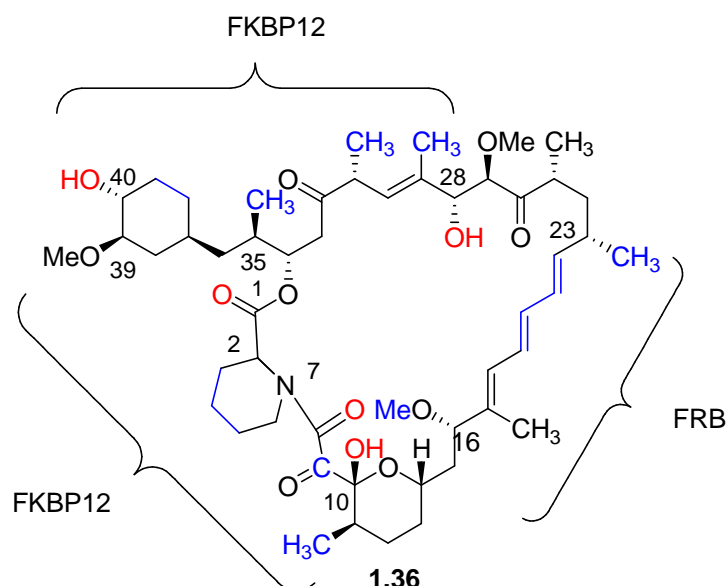


Figure 1.9: Rapamycin with regions extensively in hydrophobic contacts with FKBP12 and FRB indicated in blue, and in red atoms involved in hydrogen bonding with FKBP12.

Choi observed a slight change in conformation of rapamycin in the binary complex compared to the ternary complex. The triene arm is usually planar with the 3 double bonds conjugated but in the ternary complex the conjugation is slightly lost with a rotation of -15° between C18 and C19 and about 37° between C20 and C21, allowing the part of the molecule containing the triene to go deeper in the hydrophobic pocket of the FRB domain. The most buried part of the molecule in FRB is the methyl group on C23 that goes into a small crease between a Phe2108 and Leu 2031 of the FRB domain (**Figure 1.8 and 1.9**).

Choi believes rapamycin's macrocyclic loop, which is eight carbons longer than FK506's macrocycle, and its triene arm, reduce the conformational flexibility of the loop. This difference with FK506 is believed to be responsible for rapamycin's capacity to bind in a deep pocket of FRB compared to FK506 that does not bind to FRB.²⁶

Rapamycin interacts with FRB through close contacts with aromatic residues, and a series of interactions along the triene arm of rapamycin from C16 to C23, but does not establish any hydrogen bonds with FRB. The hydrophobic interactions concern the methyl of the methoxy group on C16, the methyl group on C23 and the alkenyl groups on C19, 20, 21, 22 of the triene. Thirty three percent of the solvent accessible surface area of rapamycin participates in the interaction with the FRB domain.²⁶

2.3.2 Interactions between both proteins.

Rapamycin has numerous binding interactions with FKBP12 and FRB but very few interactions were observed between FKBP12 and FRB, in the ternary complex from the crystal structure. Choi identified two regions of the complex showing interaction between both proteins the 40s loop of FKBP12 with the $\alpha 4$ helix of FRB, and the 80s loop of FKBP12 with the $\alpha 1$ $\alpha 2$ region of FRB. In the first region Choi found that the hydroxyl group of Tyr2105 from FRB and the oxygen of Lys47 from FKBP12 make a short contact. There is also a water-mediated salt bridge. In the second region Choi discovered that the amine group of Arg2042 from FRB makes short contacts with the hydroxyl group of Thr85 and the oxygen of Gly86 of FKBP12, and there are two water mediated interactions. In that same region Choi also found that FKBP12's structure differs from the binary complex in the 80's loop with a major change around Ile90 with both side chains and main chain angle deviating. This deviation moves the FKBP12 away from the FRB domain showing a possible repulsion between both proteins.

In order to measure the binding constants and have a better understanding of the interactions between FKBP12, FRAP and rapamycin Banaszynski²⁷ and coworkers ran a series of experiments using 3 different physical techniques: Fluorescence Polarization Assays, Surface Plasmon Resonance and 3D NMR experiments. Previously the dissociation constant between rapamycin and FKBP12 had been measured by Schreiber and coworkers $K_d = 0.2$ nM.²⁸ The binding constants were obtained independently using two different types of methods.

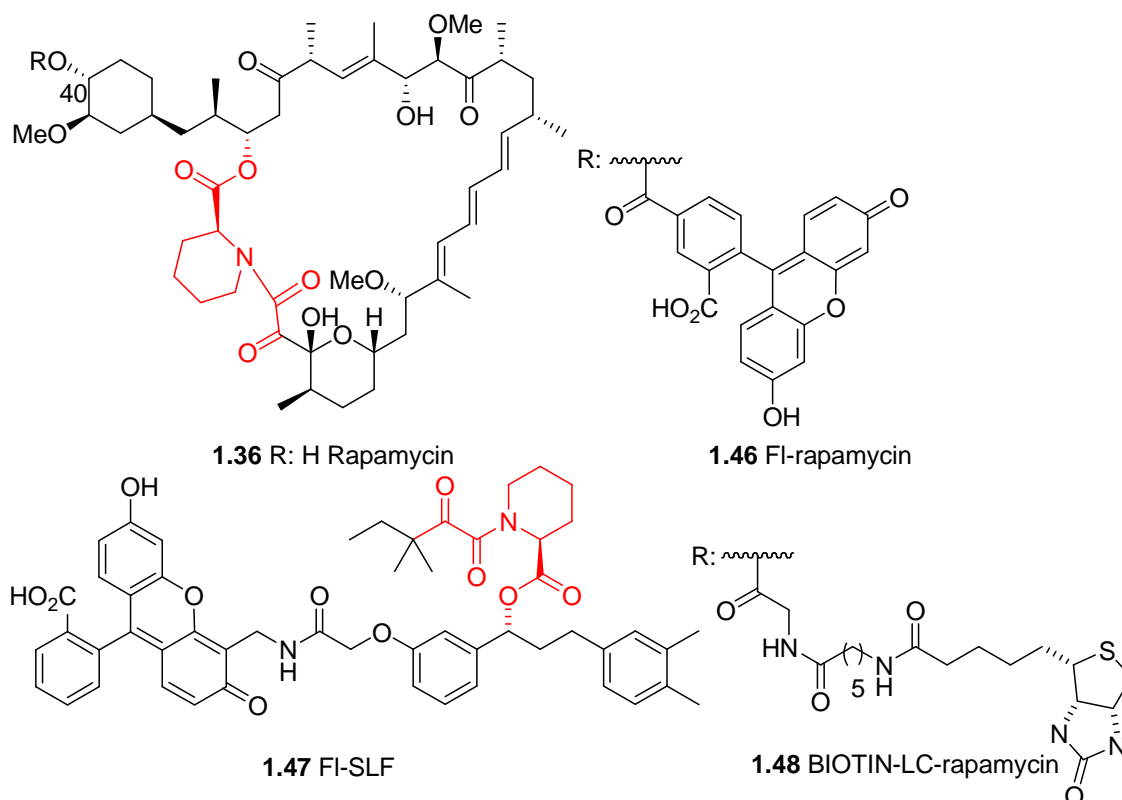


Figure 1.10: Rapamycin analogues used in the experiments of Banazynski.²⁷

Fluorescence Polarization Assays involve the excitation of fluorescent molecules, and the detection of the resulting fluorescence polarization signals emitted by those molecules, allowing them to be quantified. The alcohol group on the C40 of rapamycin was esterified with a fluorescein group. This hydroxyl on C40 was chosen as it is found on one of the most distant parts of the molecule from FRB in the crystal structure of the tertiary complex. The new rapamycin tracer was named FI-rapamycin **1.46** (**Figure 1.10**). A fixed concentration of FI-rapamycin was added to various concentrations of FRB, and formation of the FI-rapamycin FRB complex was quantified by measuring the increase in polarization. Once FRB was complexed with FI-rapamycin various concentrations of rapamycin were introduced in order to establish a competition binding experiment. The substitution of FI-rapamycin with rapamycin in the binary complex involving FRB will correspond to a decrease in polarity. The results were only partial because of rapamycin solubility issues. The partial data was fitted to a mathematical model, the best fit corresponding to K_d rapamycin-FRB = 5.2 μ M. The same studies were repeated adding FKBP12 in order to establish the binding constant of FKBP12-rapamycin with FRB. The competition results were incomplete because the fluorescein group on C40 interferes with the binding to FKBP12 as the hydroxyl group on C40 of rapamycin is involved in hydrogen bonding with FKBP12 and both methylene groups

of C41 and C42 are involved in hydrophobic interactions with FKBP12. However using another mathematical fit and considering the previous result K_d rapamycin-FRB = 5.2 μ M as a primary dissociation constant, they found as a secondary dissociation constant K_d FKBP12rapamycin-FRB = 6.2nM.

In order to validate the method and the mathematical fits the K_d of FKBP12-rapamycin had to be measured. This could not be achieved with FI-rapamycin as previously because of the proximity of the fluorescein group to the cyclohexyl moiety which is involved in the bonding to FKBP12. A molecule was synthesized which included a fluorescein moiety spaced from a diketone linked to a pipercolinyl ring (in red **Figure 1.10**) and was named FI-SLF **1.47** (**Figure 1.10**). The diketone and the pipercolinyl ring are common to rapamycin and are involved in the binding to FKBP12. The pipercolinyl ring as previously reported in the crystal structure²⁶ is the most buried portion of rapamycin in FKBP12. The experiments used FI-SLF in competition with rapamycin to bind FKBP12. After using their mathematical model the best fit was K_d FKBP12-rapamycin = 0.35nM, this was in the same range as the value previously found in the literature 0.2 nM.²⁸

In order to confirm these results Surface Plasmon Resonance (S.P.R.) was used to establish the different dissociation constants. S.P.R. measures the adsorption of molecules and proteins to a surface. The experiment started by immobilizing rapamycin molecules on separate chips and exposing them to a flow of FRB measuring the change in adsorption. In order to immobilize rapamycin a Biotin-LC moiety was fixed on the hydroxyl group of C40. Biotin-LC-rapamycin **1.48** (**Figure 1.10**) was fixed to a Neutravidin, which was itself fixed to a chip via amine coupling. The LC part, an aminohexanoic acid spacer, is there to limit the possible interactions between FRB and Neutravidin. After using Bio evaluation software analysis on the results, Banaszynski and coworkers found that K_d rapamycin-FRB was $26 \pm 0.8 \mu$ M.

In order to measure the affinity of FRB to FKBP12, FKBP12 to rapamycin and FKBP12-rapamycin to FRB, FKBP12 had to be immobilized on separate chips. A Glutathione S-transferase-FKBP12 (GST-FKBP12) fusion protein was fixed to an anti-GST antibody immobilized on the dextran surface of the chip. The interactions between FRB and FKBP12 were too small to be detected. After using the Bio evaluation analysis software the value of K_d rapamycin-FKBP12 was found to be 0.27 kDa very similar value to the previous results. In order to calculate the affinity of FRB to the rapamycin-FKBP12 binary complex, the immobilized FKBP12 was saturated with rapamycin and

then later exposed to FRB. It was found that $K_{d\text{FKBP12rapamycin-FRB}}$ was 12 ± 0.8 nM.

The final set of experiments carried out by Banaszynski were 3 dimensional NMR correlating experiments using ^{13}C and ^{15}N labeled FRB exposed to different concentrations of rapamycin and FKBP separately. Correlating those results with the crystal structures²⁶ suggested that Van der Waals interactions exist between rapamycin and FRB as a binary complex and confirmed that the interactions between both proteins without rapamycin were minimal.

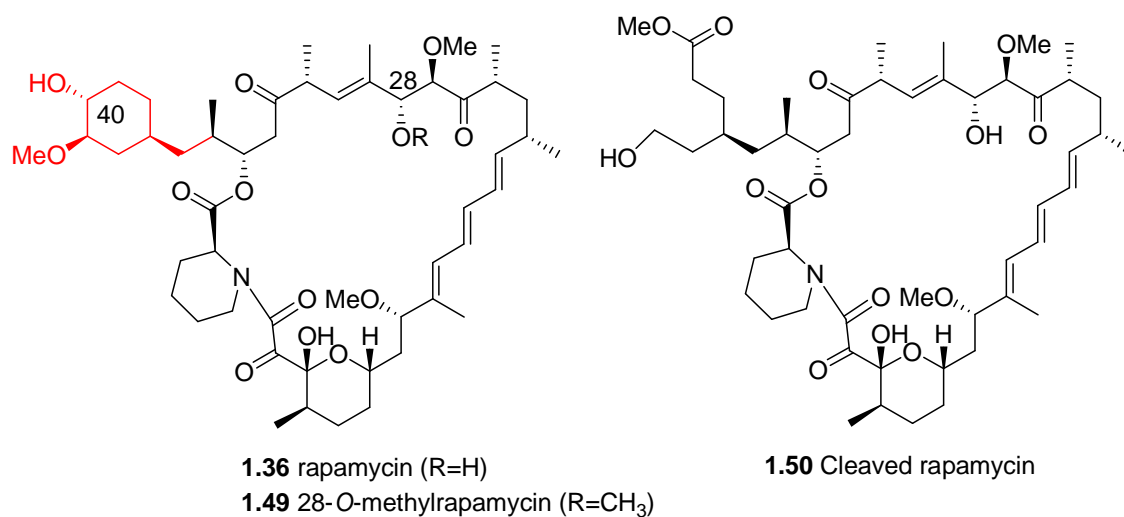
Both Fluorescence Polarization Assays and Surface Plasmon Resonance show very similar results on the binding of FKBP12 to rapamycin which is close to the previous literature value,²⁸ suggesting that they are reliable methods for establishing binding constants. Banaszynski found that FRB binds to rapamycin but the affinity is too small for them to bind in physiological conditions. From the NMR experiments they established that stabilizing Van der Waals interactions between both FRB and rapamycin existed. In the first experiment FRB was bound to rapamycin-FKBP12 roughly a thousand fold more than with just rapamycin on its own and the second experiment demonstrated a 2000 fold increase in binding. The second experiment also showed that the interactions between FRB and FKBP12 without rapamycin were negligible and this was confirmed by the NMR experiments. Banaszynski concluded that this 1000 to 2000 increase in binding of rapamycin with FKBP12 to FRB compared to rapamycin on its own with FRB suggests strong stabilizing interactions between both proteins in the ternary complex. Although Choi and coworkers from the crystal structure of the ternary complex had only found 2 zones of interactions between both proteins in the ternary complex and found some possible repulsions, Banaszynski's experiments suggest many more stabilizing interactions than destabilizing ones between both proteins in the tertiary complex.

2.3.3 Chemical modification on rapamycin and consequences on its binding and activity.

Sedrani, Kallen and coworkers²⁹ methylated the hydroxyl group on C28 of rapamycin. They ran competitive binding assays between 28-*O*-methylrapamycin **1.49** and rapamycin **1.36** (Figure 1.11) toward FKBP12, and were surprised to find that the binding of the new analogue to FKBP12 had not significantly reduced compared to

rapamycin. The hydroxyl on C28 of rapamycin is usually involved in hydrogen bonding with Glu54 of FKBP12 (**Figure 1.8**). Kallen expected that the potential loss of at least one of the 5 hydrogen bonds involved between rapamycin and FKBP12 and the sterics of the methyl group in proximity to FKBP12 would affect the binding in a more severe manner. 28-*O*-Methylrapamycin **1.49** was then tested for its immunosuppressive activity. It was found to be reduced of a 1000 fold compared to rapamycin, although the binding was hardly affected. A crystal structure of the new analogue bound to FKBP12 was then obtained and compared to the crystal structure of the binary complex FKBP12-rapamycin. Sedrani found that the conformation of the methoxy group on C40 had hardly changed compared to the previous hydroxyl group of rapamycin. The rest of the macrocycle was also unchanged except for the orientation of the cyclohexyl moiety. Two hydrogen bonds involving hydroxyl groups in rapamycin disappeared because of the C28 hydroxyl becoming a methoxy and the different orientation of the cyclohexyl moiety including the hydroxyl group on C40 that usually establishes a hydrogen bond with Gln 53 of FKBP12. The loss of hydrogen bonding with the methoxy group on C28 was expected as it was previously a hydroxyl group, but the loss of hydrogen bonding of the hydroxyl group on C40 and the shift in orientation of the cyclohexyl moiety was not expected. Sedrani had two possible explanations for the loss in activity. The first was due to an unfavourable steric interaction of the cyclohexyl ring, in its new position with FRAP. The alternative one was that the cyclohexyl moiety and possibly the ethyl side chain could play a functional role in target recognition, making it part of the effector domain.

In order to discriminate between the two hypotheses Sedrani oxidatively cleaved the cyclohexyl moiety of rapamycin (**Figure 1.11**).³⁰ Hydroxyls at position 28 and 40 of rapamycin **1.36** were first silylated, the 40-*O*-triethylsilyl protecting group was then selectively removed and the resulting free hydroxyl was oxidised to the ketone using a Swern oxidation, then oxidised to the lactone-acetal through a Baeyer-Villiger oxidation. The ring was then cleaved by mild acidic hydrolysis leading to the acid-aldehyde. The acid was then esterified, and the aldehyde selectively reduced and finally the triethylsilyl protecting group of the hydroxyl in position 28 was removed resulting in the cleaved rapamycin **1.50** (**Figure 1.11**).³⁰



In red part of the molecule involved in major conformational changes when 28-O-methylrapamycin binds to FKBP12

Figure 1.11: 28-*O*-Methylrapamycin, rapamycin and oxidatively cleaved rapamycin.

The cleaved rapamycin **1.50** was found to have a 6-fold loss in binding with FKBP12 compared to rapamycin, but its immunosuppressive activity was reduced by 380 fold. This loss of activity could be due to the decreased affinity between the FKBP12-rapamycin complex and FRAP. This loss of affinity cannot be explained by steric factors. The new cleaved cyclohexyl ring is not more hindering than the cyclohexyl moiety. Sedrani deduced from the crystal structure of the tertiary complex, by Choi that the cleaved cyclohexyl moiety should fit between FKBP12 and FRB without disturbing the formation of the ternary complex. The most plausible explanation is that part of the cyclohexyl ring is necessary to bind strongly enough to FKBP12 so that it can strongly bind to the FRB domain and inhibit FRAP. The efficacy of rapamycin in binding proteins FKBP12 and FRAP is postulated to be linked to the compounds immunosuppressive activity. Sedrani postulated that the cyclohexyl moiety is probably part of the effector domain. The hydroxyl group on C40 of rapamycin is involved in hydrogen bonding with Gln 53 of FKBP12 and both methylene groups of C41 and C42 are involved in hydrophobic interactions with FKBP12.

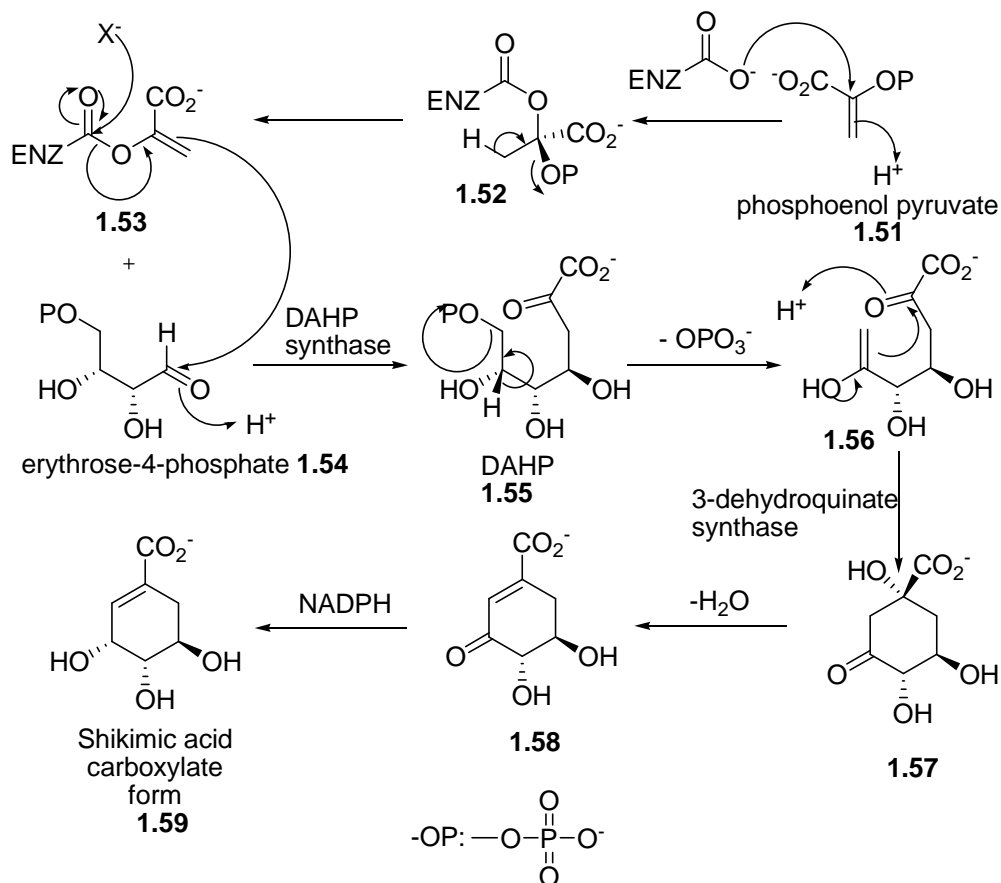
3 BIOSYNTHESIS OF RAPAMYCIN

3.1 Starter Unit of the Polyketide Synthesis

3.1.1 The shikimic acid pathway

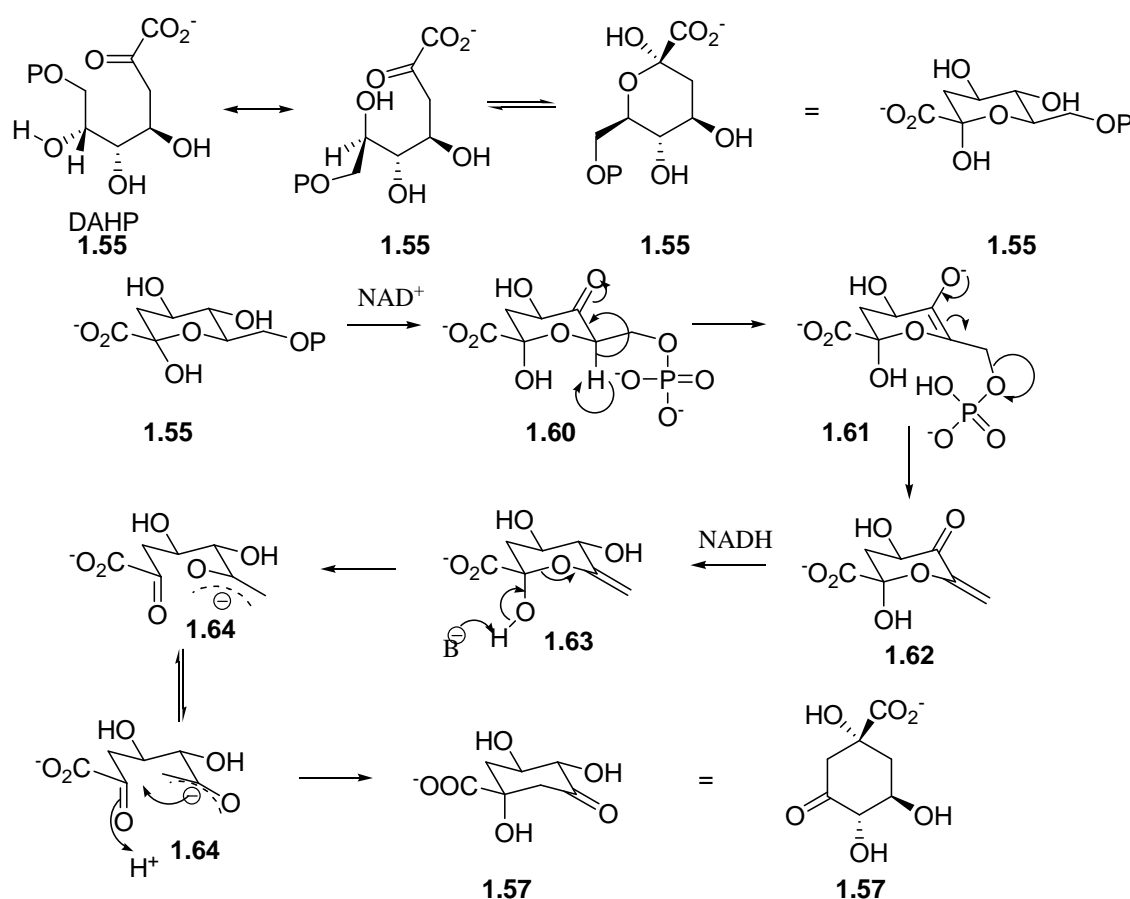
The main structure of the rapamycin macrocyclic ring is derived from polyketide biosynthesis, using as a starter unit (4*R*, 5*R*)-4,5-Dihydroxy-cyclohex-1-enecarboxylic acid derived from the shikimic acid pathway.

In the first step catalysed by 3-deoxy-*D*-arabino-heptulosonate-7-phosphate (DAHP) synthase, phosphoenol pyruvate **1.51** reacts with erythrose-4-phosphate sugar **1.54** to produce 3-deoxy-*D*-arabino-heptulosonate-7-phosphate DAHP **1.55** (Scheme 1.4).³¹ Isotopically labelled enol pyruvate experiments indicated that the P-O bond of phosphoenol pyruvate **1.51** is first substituted by a C-O bond before being cleaved (Scheme 1.4).^{31, 32} The enzyme, through a carboxylic group, performs a nucleophilic addition on the most substituted part of the double bond to produce intermediate **1.52**. Then the phosphate group eliminates to form the enzyme associated oxiacrylate **1.53**.



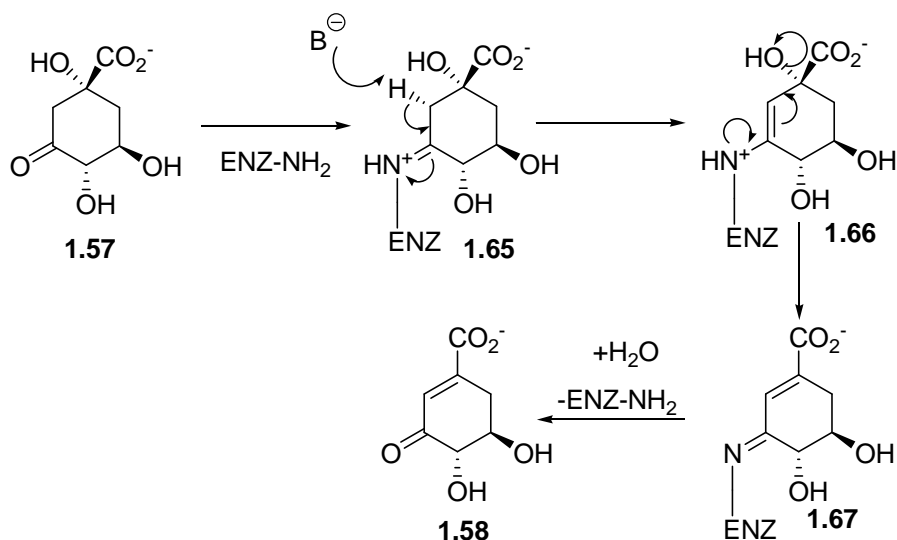
Scheme 1.4: Shikimic acid biosynthesis.³¹

In the second step DAHP **1.55** dephosphorylates to intermediate **1.56** and then cyclises to 3-dehydroquinate **1.57**. Knowles suggested a mechanism for step 2 involving DAHP **1.55** in its hemi-ketal form (Scheme 1.5).^{31, 33} In this mechanism one of the hydroxyl group's of hemi-ketal **1.55** is oxidised to ketone **1.60** by NAD^+ . The phosphate in **1.60** acts as an internal base and abstracts the proton on the carbon in alpha of the ring oxygen (**1.61**), facilitating the elimination of the phosphate group (**1.62**). The ketone of **1.62** is reduced back to the alcohol of **1.63** by NADH . After a base deprotonation of the alcohol on the carbon in between the double bond and the carboxylate group, the ring opens (**1.64**), and rearranges to form 3-dehydroquinate **1.57** after cyclisation (Scheme 1.5).



Scheme 1.5: Plausible mechanism between **1.55** and **1.57** proposed by Knowles.^{31, 33}

The third step transforms 3-dehydroquinate **1.57** into 3-dehydroshikimate **1.58** through a syn elimination of water. The suggested mechanism by Man (Scheme 1.6) starts with the condensation of 3-dehydroquinate **1.57** by an enzyme forming the imine **1.65**.

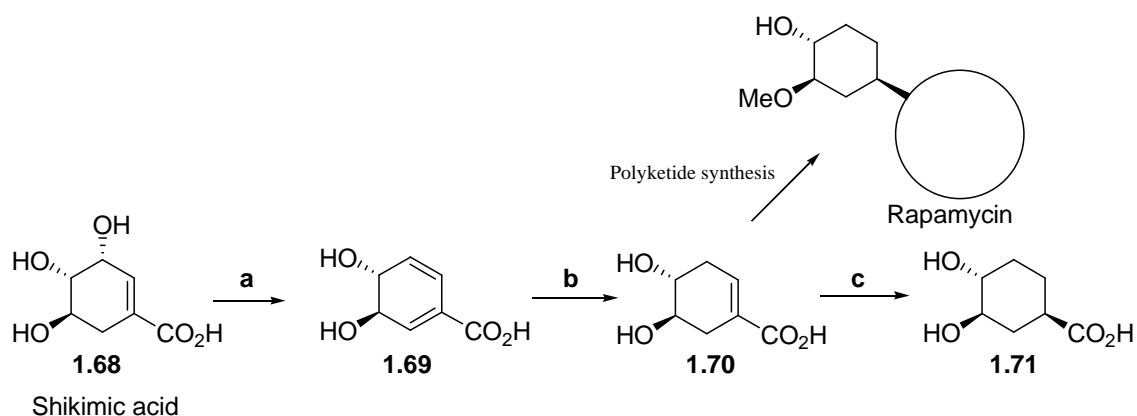


Scheme 1.6: Suggested mechanism for the syn elimination of water by Mann.^{31, 34}

A base-mediated deprotonation of the proton alpha to the imine, is followed by a rearrangement to form **1.66**, followed by the elimination of a hydroxide to form **1.67**. Finally the enzyme is hydrolysed and 3-dehydroshikimate **1.58** is obtained (**Scheme 1.6**). The final step is the reduction of the ketone of 3-dehydroshikimate into the alcohol yielding shikimic acid in its carboxylate form **1.59** (**Scheme 1.4**).

3.1.2 Route from shikimic acid to the determination of the true starter unit

The shikimic acid pathway has been well defined. Shikimic acid can undergo various biosynthetic conversions into aromatic amino acids and secondary metabolites. Previously Floss and coworkers³⁵ had investigated the biosynthesis of the cyclohexanecarboxylic acid starter unit of ω -cyclohexyl fatty acids in *Alicyclobacillus acidocaldarius* which is also derived from shikimic acid. The first step of the starter unit from shikimic acid toward cyclohexanecarboxylic acid was identified as the first step toward rapamycin's starter unit.³⁶ After isotopic incorporating experiments Reynolds and coworkers identified the rest of the pathway leading to the starter unit of rapamycin and FK506.³⁷ This was confirmed by further isotopic incorporation experiments led by Leadlay, Staunton and coworkers the stereochemistry of the mechanism was defined (**Scheme 1.7**).^{9, 36}



a: Stereospecific 1,4 anti-elimination of water b: syn reduction of the double bond and isomerisation of the remaining double bond from and an anti reduction c: reduction of the final double bond.

Scheme 1.7: Biosynthetic pathway to DHCHC **11** in FK520 and rapamycin.³⁶

The first step **a** is the 1,4 anti- conjugate elimination of water from shikimic acid **1.68** to yield 3,4-dihydroxy-cyclohexa-1,5-dienecarboxylic acid **1.69**, this is followed by the syn reduction of the double bond and a suprafacial 1,3-allylic rearrangement to yield 4,5-dihydroxy-cyclohex-1-enecarboxylic acid **1.70**. Finally an anti reduction of the conjugated double bond was established to yield (1*R*, 3*R*, 4*R*)-3,4-dihydroxycyclohexanecarboxylic acid **1.71** (**Scheme 1.7**).

Leadlay, Staunton and coworkers defined the stereochemistry of the mechanism involved in step **a** (**Scheme 1.7**) by feeding stereospecifically deuterated shikimic acid **1.72** and **1.73** (**Figure 1.12**). No deuterated rapamycin was produced by feeding **1.72**, but deuterated rapamycin was obtained when **1.73** was fed, proving that the pro *R* proton eliminates in anti.

In order to determine which of these molecules was the original starter unit, feeding experiments of deuterated labelled compounds were also carried by Leadlay, Staunton and coworkers⁹ on **1.68**, **1.69**, **1.70** and **1.71** (**Scheme 1.7**) deuterated analogues respectively named **1.73**, **1.74**, **1.75** and **1.76** (**Figure 1.12**). They were synthesised and fed separately to *S. hygroscopicus*.^{36, 38, 39} The specific incorporation of **1.73** was measured at 25%, **1.74** 79%, **1.75** 87%, and **1.76** 52%. **1.75** was the most incorporated and **1.70** could be the true starter unit.

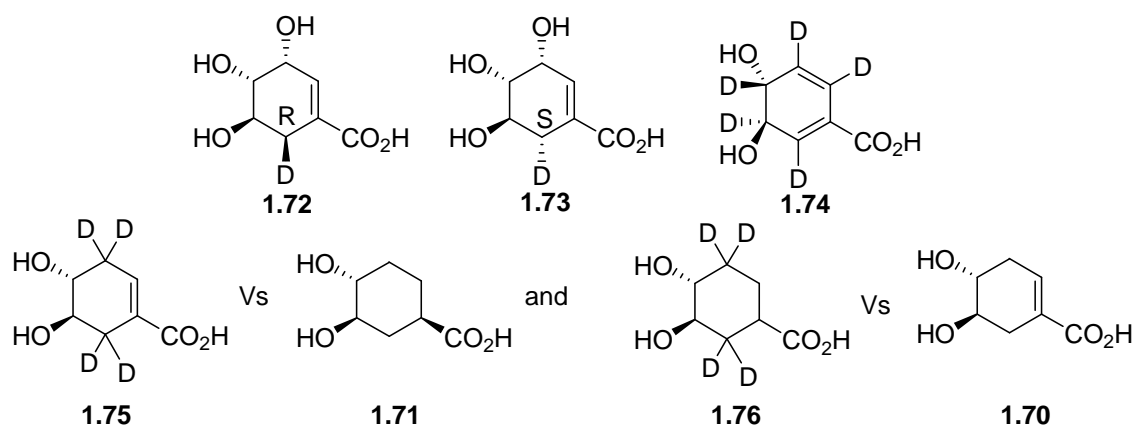


Figure 1.12: Deuterated molecules incorporated and competition experiments in *Streptomyces hygroscopicus*.

From the previous results and the structure of rapamycin only **1.70** or **1.71** can be the true starter unit for the rapamycin polyketide synthesis. In order confirm that **1.70** is the starter unit, **1.70** and **1.71** were incorporated in competition, in cross experiments between **1.75** and **1.71**, and **1.76** and **1.70** for different concentrations (**Figure 1.12**). Experiments tend to show that **1.70** has higher incorporation levels than **1.71** for identical conditions and that **1.70**, even at small concentrations compared to **1.76**, strongly inhibits the incorporation of **1.76**. The incorporation of **1.75** was not inhibited by the presence of a four times more concentrated solution of **1.71**. 4, 5-Dihydroxy-cyclohex-1-enecarboxylic acid **1.70** is the true starter unit. The observation that **1.68**, **1.69**, **1.70** and **1.71** can be directly incorporated using wild type *S. hygroscopicus* seems to indicate a broad substrate specificity that would allow the production of rapamycin analogues by feeding different starter units.

3.2 The Polyketide Biosynthesis

3.2.1 Common elements between fatty acid biosynthesis and PKS Type 1, 2, 3

Fatty acid biosynthesis and polyketide biosynthesis have strong homologies,⁴⁰ they are both used by nature for chain extension, and have a common group of precursors and catalytical domains that are used for chain assembly.

All fatty acid synthases have the same set of catalytic domains: the ketosynthase (KS), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), thioesterase (TE) and a malonyl-acetyl transferase (MAT).⁴⁰ MAT is the only domain that

is not common to polyketide synthase (PKS). Instead PKS has an acyl transferase (AT) in order to transfer various acyl units usually from one module to another. It also has CoA ligase (CoL) in order to load the starter unit. Contrary to fatty acid synthase not all those modules are involved in all PKS, and are not always on the same protein. PKS have been classified into three groups PKS I, II and III.

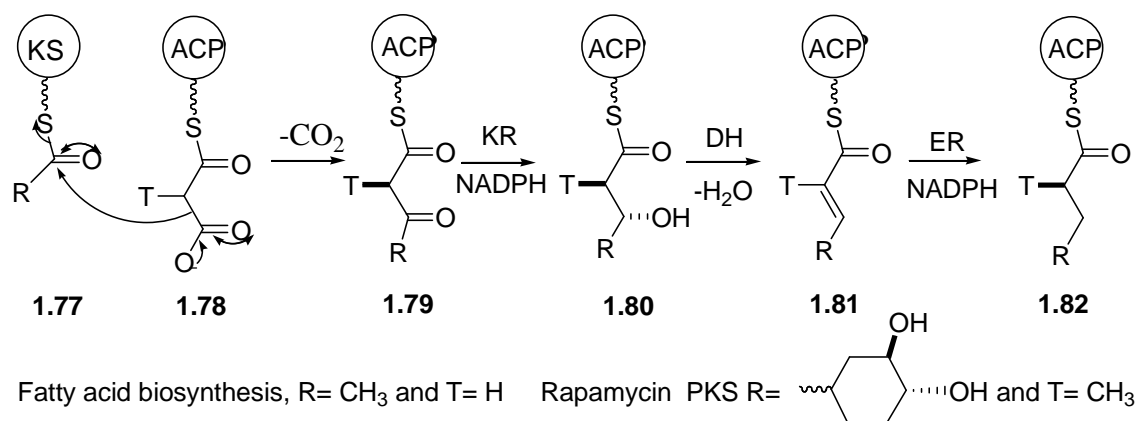
PKS I are single large multifunctional enzymes organised into modules and are ACP dependant. Each module is responsible for chain elongation. PKS I has been subdivided in modular PKS I and iterative PKS I. In modular PKS I all the modules are used successively only once. The number of chain extensions is equal to the number of modules making the biosynthesis potentially predictable. Iterative PKS I only has one module repetitively used, but the chain extension unit can vary and not all the domains are reused for each chain extension making it difficult to predict. PKS II are multienzyme complexes, each enzyme only corresponding to one domain used repetitively or not. PKS II are also ACP-dependent. PKS III are single multifunctional enzymes with multiple modules, repetitively used but does not use ACP in its cycle.

Modular PKS I due to their non iterative mechanism build more complex structures, than the other iterative PKS. Rapamycin results from modular PKS I.

3.2.2 Rapamycin's PKS

The extension cycles of rapamycin are similar to fatty acid synthesis. The first module of the rapamycin PKS once the starter unit is loaded is very similar to the fatty acid first extension cycle.

Fatty acids are built from C₂ units by repeated head to tail linkage, until a chain of required length is assembled.⁴⁰ A starter acyl on a KS domain **1.77** is condensed with a malonyl unit on an ACP **1.78** in a similar fashion to a Claisen condensation (**Scheme 1.8**). The ACP-malonyl **1.78** goes through a decarboxylation and condenses on the carbonyl of KS-acyl **1.77**, resulting in the transfer of the acyl group from KS to the ACP chain **1.79** (**Scheme 1.8**). The resulting beta ketoester of **1.79** is then reduced to the hydroxyl group of **1.80** through a keto reductase domain (KR), using NADPH as a cofactor. Catalysed by a DH domain the new hydroxyl group dehydrates and **1.81** is obtained. Finally the double bond of **1.81**, resulting from the dehydration, is reduced by an ER using NADPH as a cofactor. The resulting chain of **1.82** is then 2 carbons longer than the original **1.77** (**scheme 1.8**).



Scheme 1.8: Similarities between first extension cycle of the fatty synthase and the first cycle of chain extension catalysed by module 1 of rapamycin's polyketide synthesis.

The differences between the first extension cycle of both fatty acid synthesis and polyketide synthesis of rapamycin are the starter units and the extension units (**Scheme 1.8**). The starter unit of rapamycin being the dihydroxycyclohexyl moiety and the extender unit identified as methyl malonate (**Scheme 1.8**).

Isotopic labelled acetate and propionate units were fed to *S. hygroscopicus* by Demain and co-workers.⁴¹ The experiment identified that during the PKS assembly rapamycin incorporates seven acetate and seven propionate units in its structure (**Figure 1.13**).^{40, 41} The 7 Propionate (P) and 7 acetate (A) units are incorporated in this sequence (A): P-A-P-P-A-P-P-A-A-P-A.

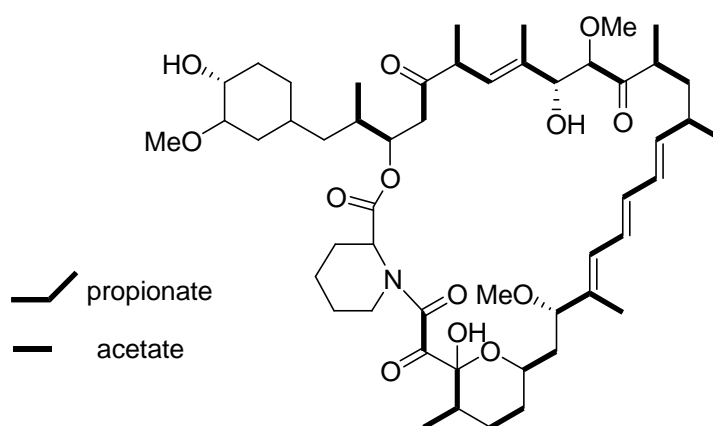
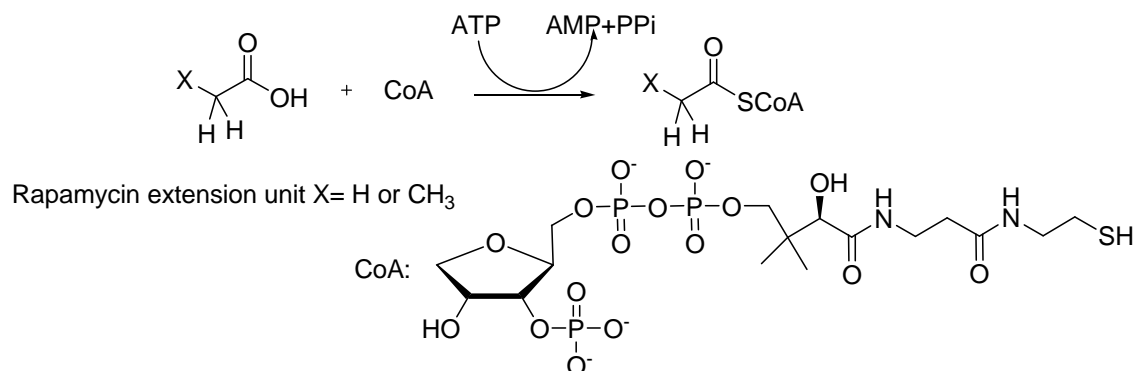


Figure 1.13: Acetate and propionate units incorporated in rapamycin's structure.

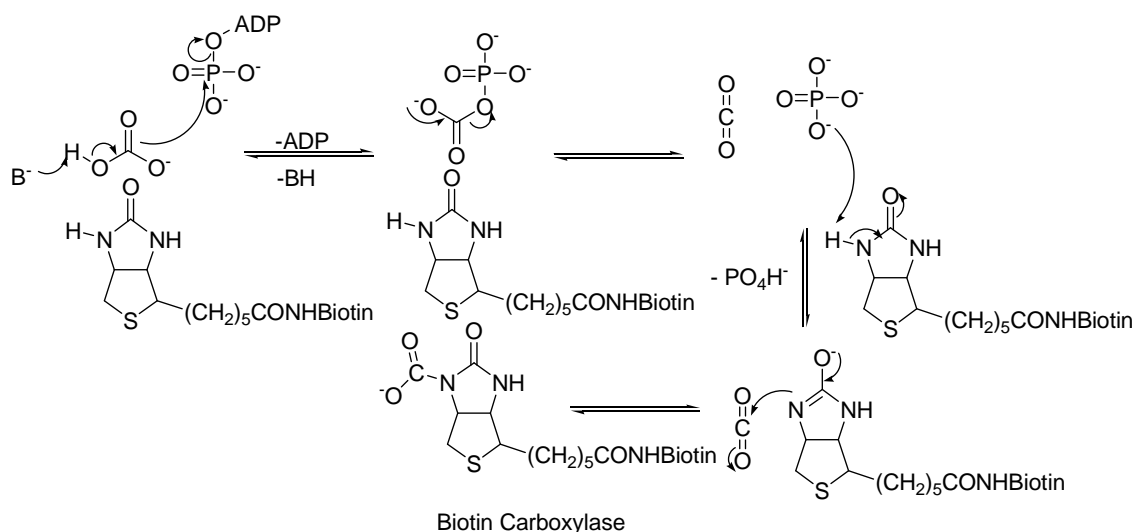
Propionate and acetate units are first carboxylated to form the more reactive malonyl and methyl malonyl CoA derivatives.

In order to achieve the carboxylation of acetate and propionate, both first associate to coenzyme A (CoA) in an ATP dependant reaction (**Scheme 1.9**).⁴²



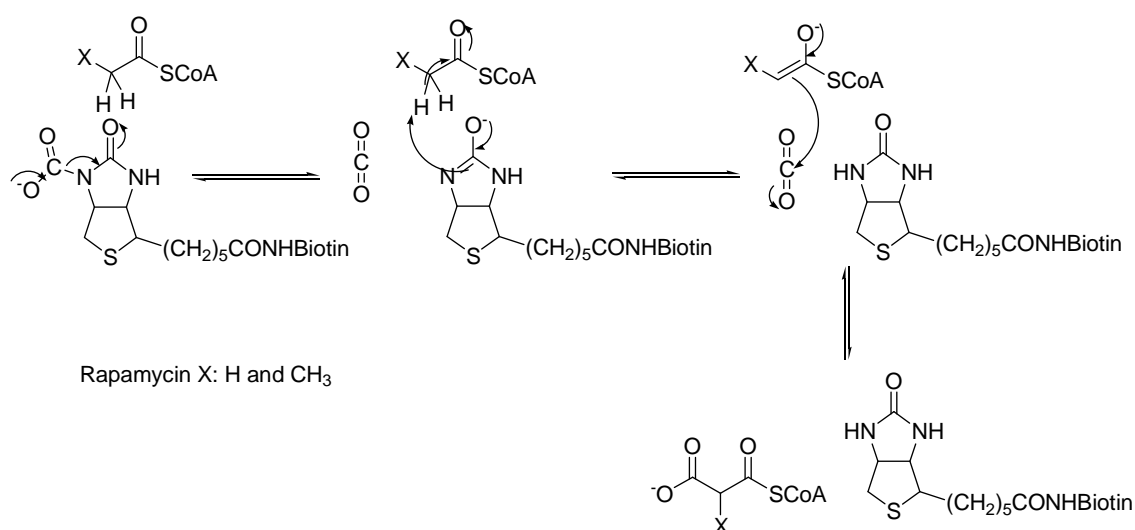
Scheme 1.9: Reaction between the carboxylic acid and CoA.

Once associated with CoA the starter units can be carboxylated through a carboxylase enzyme (acetyl-CoA carboxylase or propionyl-CoA carboxylase). Those enzymes are biotin-dependant, and have two catalytic activities: -biotin carboxylase and carboxyltransferase. The first uses a carbonate to produce CO₂ and biotin in order to trap it as a carbamate. A pyruvate group from ATP is also involved in the mechanism (**Scheme 1.10**).



Scheme 1.10: Biotin carboxylase trapping CO₂ from carbonate.⁴²

The second catalytic role is as a carboxyltransferase. The carboxylated biotin releases CO₂ and acts as a base with the acyl-CoA allowing it to condense on the CO₂ transforming acetyl-CoA into malonyl-CoA and propionyl-CoA into methylmalonyl-CoA (**Scheme 1.11**). These substrates undergo decarboxylative condensation mediated by the PKS.



Scheme 1.11: Carboxyltransferase releasing CO₂ and carboxylating the acetylCoA

The organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus* was determined by Leadlay, Staunton and coworkers.⁴³ The different domains identified in the rapamycin PKS are CoA ligase (CoL), enoyl reductase (ER), acyl carrier protein (ACP), ketosynthase (KS), ketoreductase (KR), dehydratase (DH), and acyltransferase (AT). No thioesterase (TE) is involved. In total fourteen modules were identified. Each module contains three domains required to catalyse one cycle of chain extension KS, AT, ACP, as well as a variable set of the other 4 domains.

The structural genes responsible for rapamycin polyketide synthase are a cluster formed by three very large open reading frames encoding the multi enzymes RAPS 1 (900 kDa, 4 modules), RAPS 2 (1.07 MDa, 6 modules) and RAPS 3 (660 kDa, 4 modules). In total the three proteins contain 70 catalytic functions, making this one of the most complex multienzyme systems identified in nature (**Figure 1.14**).⁴⁰

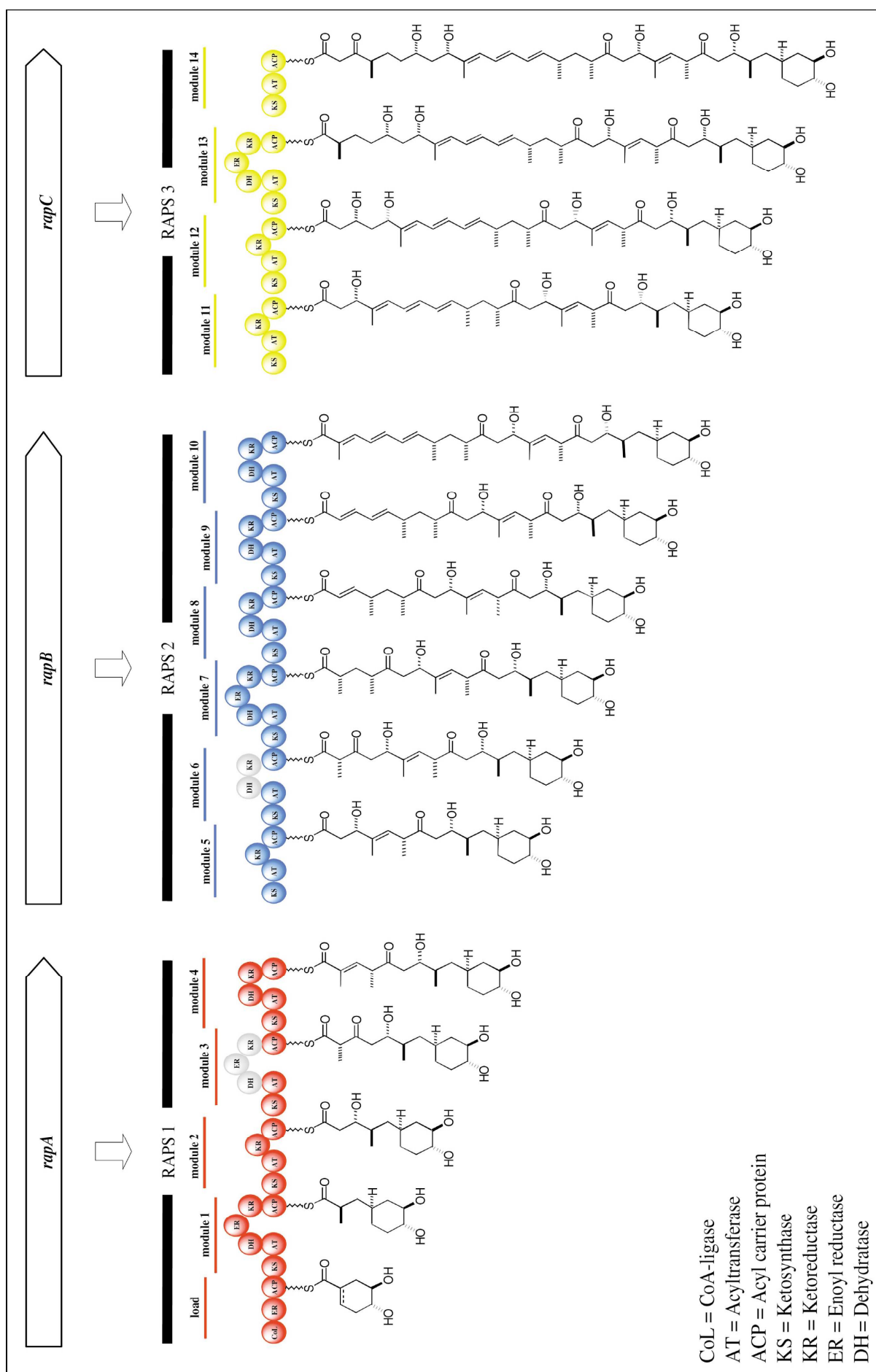


Figure 1.14: Domain organisation of PKS by Wiseman and Staunton.⁴⁰

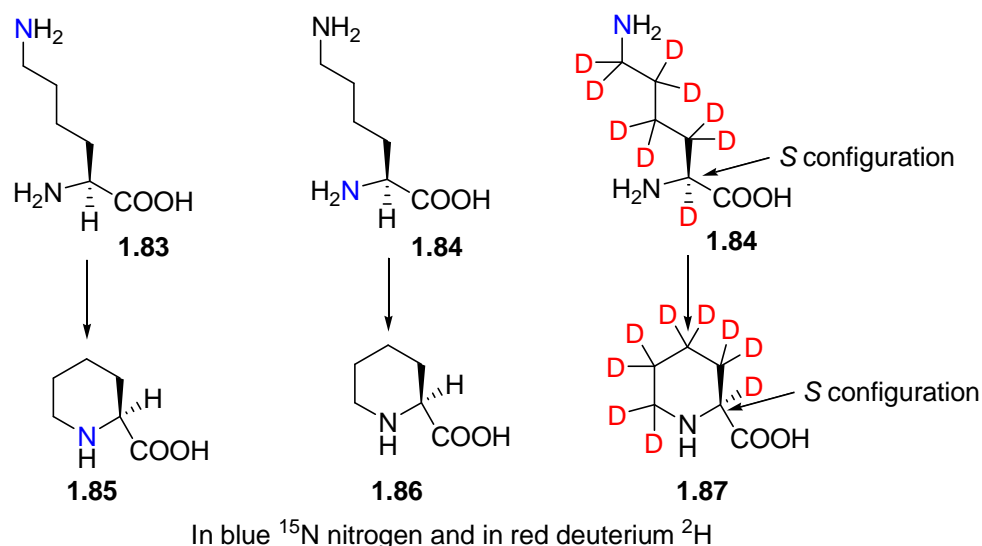
The domain in grey in **Figure 1.14**, DH, ER, KR in module 3, and DH and KR in module 6 all appear to be active but do not intervene in the polyketide chain synthesis. Staunton suggested a possibility that these sites are involved in a later oxidation of the molecule after the macrocycle has formed.⁴⁰ Before the first extension, *rapA* encodes for 3 domains CoL (CoA-ligase), ER and ACP. These load the starter unit and probably reduce the double bond of the starter unit.⁴⁴ As it has been previously established by Leadlay, Staunton and coworkers³⁶ the true starter unit still had a double bond. At the end of module 14 the pipercolic ring is still not featured in the structure.

3.3 The Pipercolic Ring

Leadlay and coworkers¹⁰ produced a modified *S. hygroscopicus* strain suppressing a gene named *rapL* L-lysine Cyclodeaminase, believed to be responsible for the formation of the pipercolic ring in rapamycin. The production levels of rapamycin were very low, but once exogenous pipercolic acid was fed the production levels went back to wild type levels. This experiment demonstrates that pipercolic acid is the precursor of the pipercolic ring in rapamycin, and that *rapL* is responsible for the synthesis of pipercolic acid.¹⁰

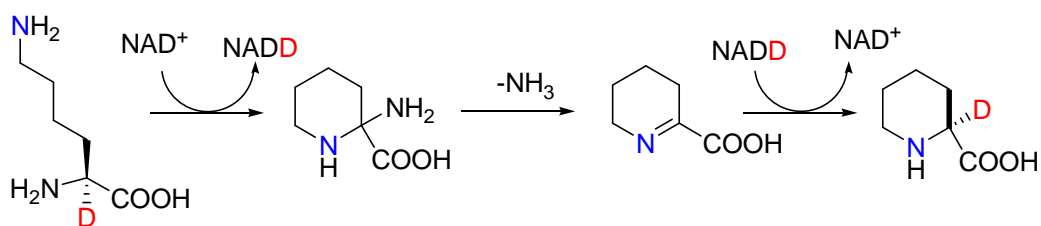
Paiva and coworkers fed exclusively isotopically labelled L-¹⁴C-lysine and D,L-³H-pipercolate in separate experiments to *S. hygroscopicus* cultivated in medium deprived of natural lysine.⁴⁵ They observed the production of isotopically labelled rapamycin in both cases, proving that pipercolic acid is the origin of the pipercolic ring in rapamycin and that lysine is the precursor of pipercolic acid in the rapamycin biosynthesis.

Walsh and coworkers later expressed *rapL* coding for L-lysine Cyclodeaminase in *E. coli*. Walsh observed lower levels of NAD⁺ in the overexpressed *rapL* compared to the normal levels in rapamycin biosynthesis.⁴⁶ In order to understand the mechanism of the formation of pipercolic acid from lysine Walsh fed different types of isotopically ¹⁵N and ²H labeled lysines, with extra NAD⁺ to L-lysine Cyclodeaminase in separate experiments. Labeled lysines **1.83**, **1.84** and **1.85** were fed and the corresponding pipercolic acids **1.86**, **1.87** and **1.88** were obtained (**Scheme 1.12**).



Scheme 1.12: Feeding experiments by Walsh and coworkers.⁴⁶

The results indicated that the nitrogen present on the ring of pipercolic acid originates from the amine on the ϵ -carbon of L-lysine. The stereochemistry on the α -carbon was retained. Walsh proposed the mechanism below (**Scheme 1.13**).⁴⁶



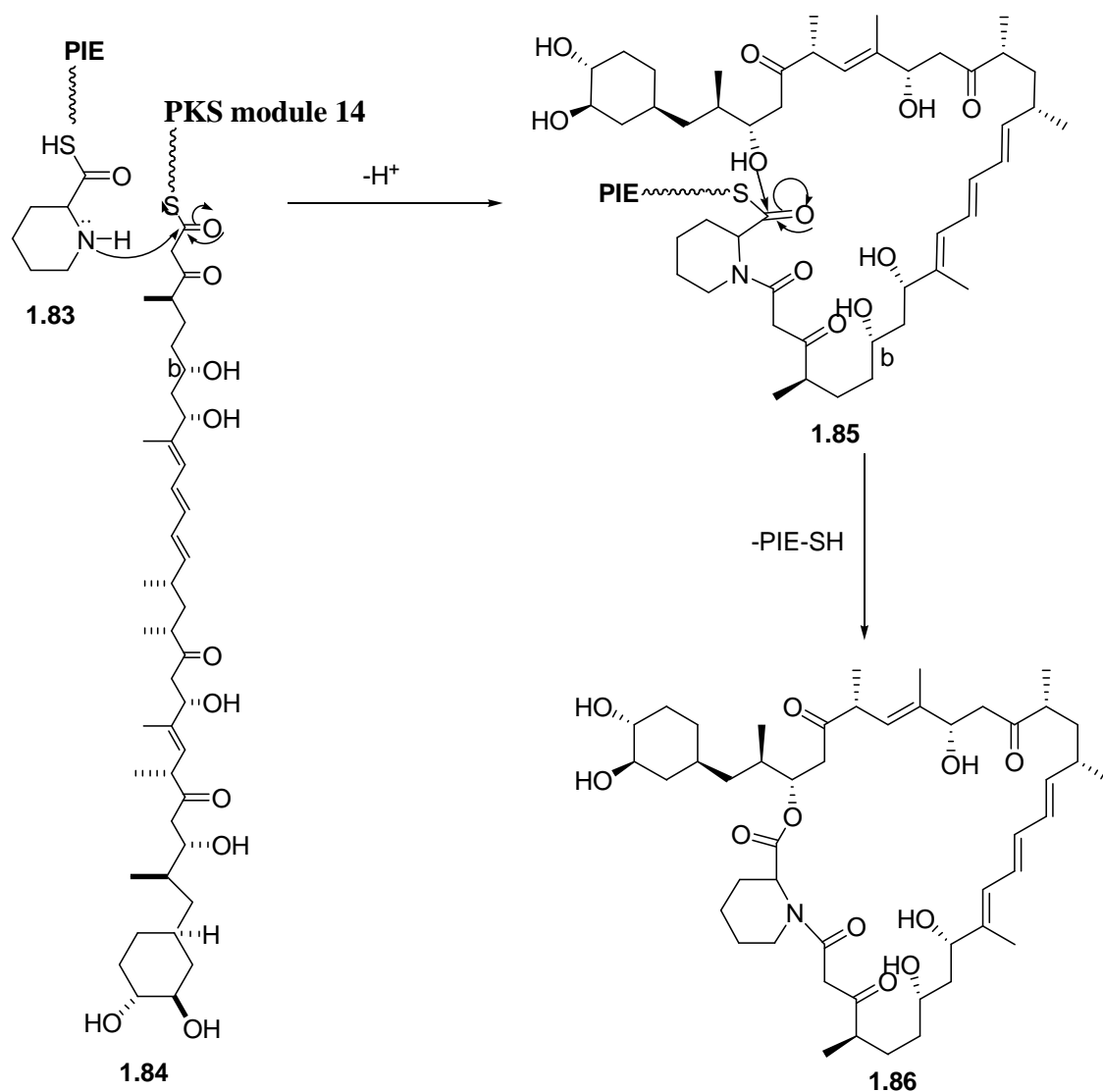
Scheme 1.13: Mechanism proposed by Walsh merging the results of the labelled incorporation experiments.^{46, 47}

The amine performs a nucleophilic attack on the carbon α to the carboxylic acid assisted by the oxidation by NAD^+ resulting in cyclisation. The amine group eliminates as ammonia assisted by the ring nitrogen. The double bond is finally reduced by NADH restoring the hydrogen and the *S* configuration on the carbon α to the carboxylic acid (**Scheme 1.12** and **1.13**).

3.4 The Assembling of the Macrocycle and the End of the Biosynthesis of Rapamycin.

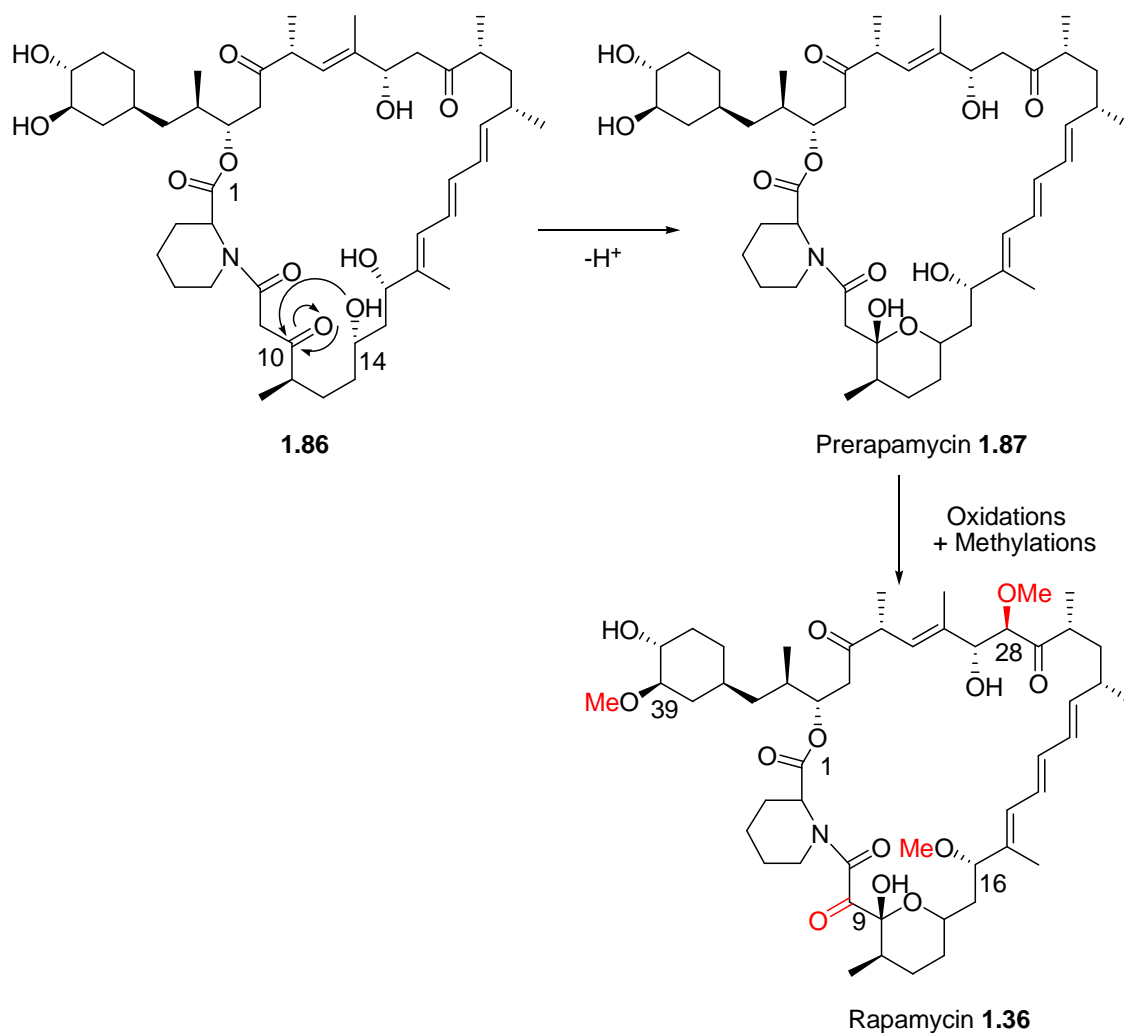
Within the polyketide synthase genes is a gene called *rapP*, this has sequence similarities with non ribosomal polypeptide biosynthesis.^{40, 48} It codes for a protein called pipercolate-incorporating enzyme (PIE). PIE is believed to catalyse the formation of ester and amide

bonds to pipecolic acid. The pipecolic acid first associates with PIE through a thioester bond. The amine group on the pipecolic ring of **1.83** attacks the carbonyl of the thioester linking the polyketide structure to PKS module 14 **1.84** releasing module 14 and linking the polyketide structure to the pipecolic thioester linked to PIE obtaining **1.85**. Next the terminal hydroxy group attacks the thioester to form the macrolide **1.86**, releasing it from PIE (Scheme 1.14).⁴⁰



Scheme 1.14: Formation of macrocyclic structure of rapamycin.⁴⁰

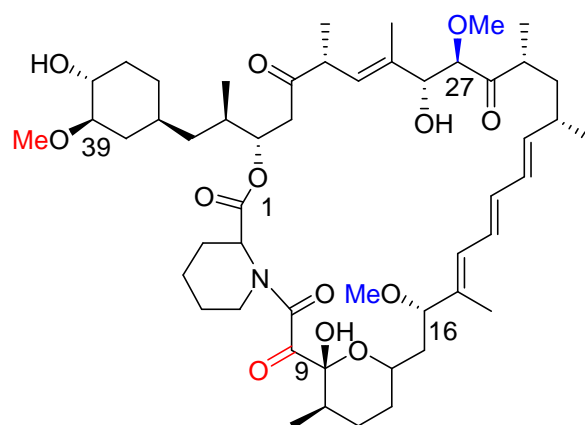
In the macrolactone **1.86** the hydroxyl on carbon 14 attacks the ketone on carbon 10 forming a 6 membered lactone.



Scheme 1.15: Final steps of the biosynthesis of rapamycin.

This molecule **1.87** is called prerapamycin and was first isolated by Leadlay and coworkers (**Scheme 1.15**).⁴⁹ They first produced a modified strain of *S. hygroscopicus* named MG-210 with the region *rapIJKLMNOQ* of the rapamycin cluster, which encode the processing genes, removed using a double recombination strategy.

At first no rapamycin related compound was produced. *S. hygroscopicus* MG2–10 was then independently complemented with full-length copies of each of the genes which had been removed using integrative expression plasmids.⁴⁹ The only productive strain was found when *rapK* was reintroduced leading to the production of prerapamycin. The strain was named *S. hygroscopicus* NRRL5491.



Black+ blue + red: Rapamycin **1.36**

Black+ red: 16-*O*-desmethyl-27-desmethoxyrapamycin **1.88**

Black: Prerapamycin **1.87**

Figure 1.15: 16-*O*-desmethyl-27-desmethoxyrapamycin, prerapamycin and rapamycin.

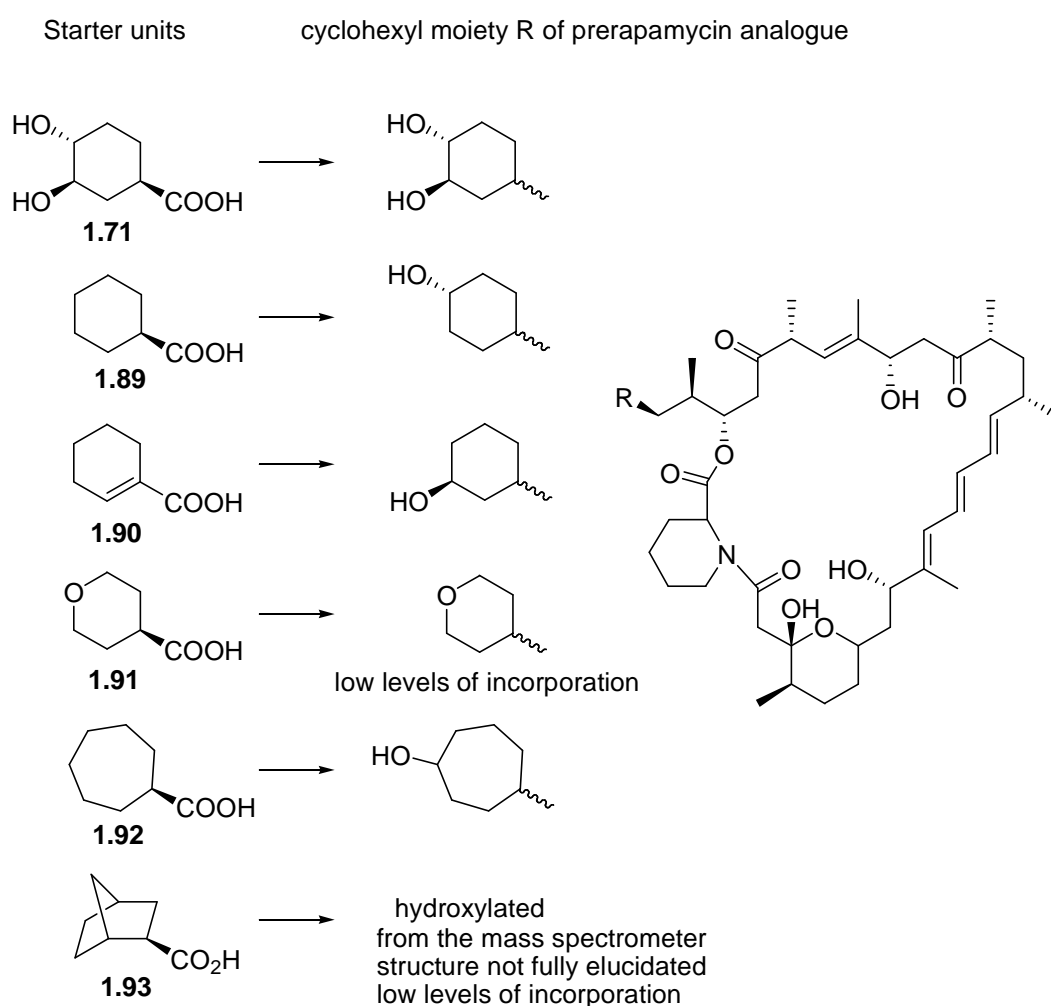
Sheridan and coworkers discovered that *S. hygroscopicus* MG-210 produced prerapamycin if the exogenous starter unit **1.71** was fed (**Scheme 1.16**). Sheridan deduced that *rapK* was responsible for the biosynthesis of the shikimic acid derivative starter unit. Previously Reeves and coworkers⁵⁰ produced 16-*O*-desmethyl-27-desmethoxyrapamycin **1.88** using a strain of *S. hygroscopicus* with *rapQONML* genes knocked out, feeding pipecolic acid because of the lack of *rapL* (**Figure 1.15**). They identified that *rapJ* and *rapN* coded for two cytochrome P450 monooxygenases responsible for oxidation of C27 and C9. *RapI*, *rapM* and *rapQ* code for three *O*-methyl transferases, probably responsible for the methylation of the hydroxyl groups on C39, C27 and C16.

Sheridan and coworkers later assigned each gene⁵¹ using MG-210, first complementing it with *rapK* and *rapL* allowing the production of prerapamycin, and then introducing separately *rapI*, *J*, *M*, [*N*, *O*], *Q* and analysing individually their production by 3 D NMR and MS. *RapN* and *rapO* were always associated as *rapO* and *rapN* are translationally fused. *RapO* codes for a ferridoxin that supports electron transfer to the cytochrome encoded by *rapN*.⁵¹

The genes were also sometimes reintroduced two by two in order to confirm the results. Twenty four analogues were obtained in this manner and structurally elucidated by MS and NMR. In order to verify the results the genes were separately expressed in *S. lividans* TK24 feeding the appropriate previous analogues were fed in order to confirm their function. Sheridan proposed that *rapI* first *O*-methylates the hydroxyl on C39. Next *rapJ* introduces the keto group on C9. *RapM* *O*-methylates the hydroxyl on C16. *RapN*

hydroxylates C27 in collaboration with *rapO*. Finally *rapQ* O-methylates the hydroxyl on C27, forming rapamycin.

Sheridan and coworkers (*Biotica Technology Ltd*) also found that different types of substrates were incorporated by MG-210. Substrates **1.71**, **1.89**, **1.90**, **1.91**, **1.92** and **1.93** were then fed and different prerapamycin analogues were obtained, showing high substrate flexibility compared to the wild type (**Scheme 1.16**). Sheridan also observed that some of the starter units **1.89**, **1.90**, **1.92** and **1.93** had been hydroxylated during the process (**Scheme 1.16**).

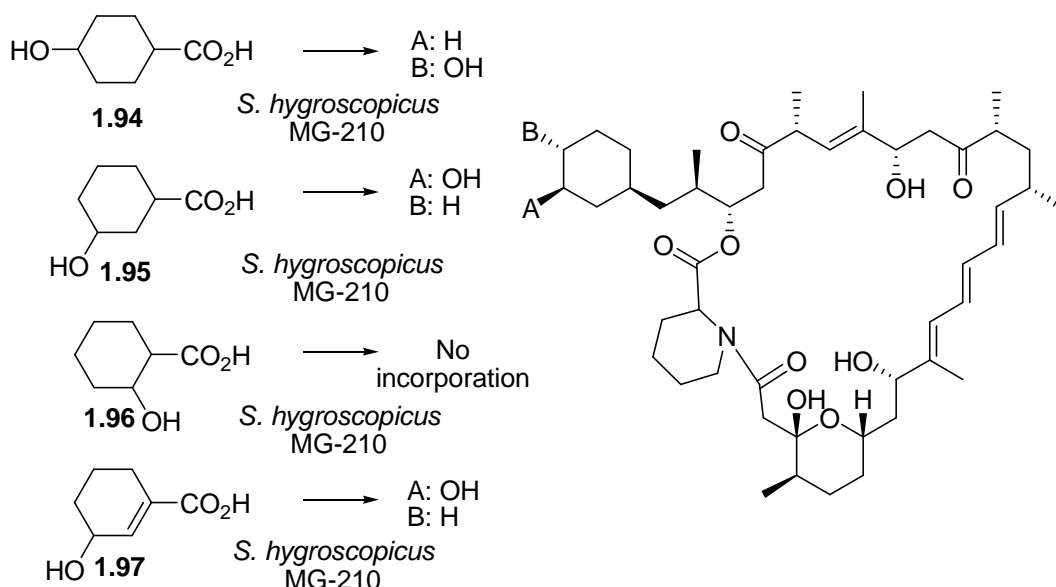


Scheme 1.16: Alternative starter units that were successfully fed by *Biotica Technology Ltd.*⁴⁴

No hydroxylation was obtained for tetrahydro-2*H*-pyran-4-carboxylic acid **1.91**. Previously the norbornane moiety **1.93** had not been incorporated by *S. hygroscopicus* wild type. The removal of competition allows greater substrate flexibility. *S. hygroscopicus* MG-210 can potentially produce a whole new range of rapamycin

analogues by feeding new starter units and also by reintroducing some genes, both techniques can be combined.

In order to have a better understanding about the hydroxylation process and the importance of the hydroxyl group for incorporation, starter units with hydroxyl groups in different positions were fed to *S. hygroscopicus*. 4-Hydroxycyclohexanecarboxylic acid **1.94**, 3-hydroxycyclohexanecarboxylic acid **1.95**, 2-hydroxycyclohexanecarboxylic acid **1.96**, 3-hydroxycyclohex-1-enecarboxylic acid **1.97** was fed to *S. hygroscopicus* MG-210 (Scheme 1.17).⁵²



Scheme 1.17: Hydroxylated starter units fed to *S. hygroscopicus* MG-210 and produced prerapamycins.⁵²

4 AIMS OF THE PROJECT

The cleavage of the cyclohexyl moiety of rapamycin by Sedrani and workers corresponded with a slight loss in binding with FKBP12 combined to a huge loss of activity in the inhibition of FRAP, and therefore a loss in its immunosuppressive activity.³⁰ This suggests that the cyclohexyl moiety of rapamycin is part of the effector domain. The crystal structure of the ternary complex by Choi and coworkers indicate that a crucial hydrogen bond is established between Gln53 of FKBP12 and the hydroxyl group on C40. This hydrogen bond is believed to be crucial to rapamycin's immunosuppressive activity. A fluorine carbon bond (1.39 Å⁰) is isosteric to a hydroxyl

group (1.43 Å), but fluorine is only a hydrogen bond acceptor compared to a hydroxyl group that is also a donor. By replacing the hydroxyl group by fluorine this could provide clues as to the origin of rapamycin's immunosuppressive activity. The presence of fluorine could also possibly enhance rapamycin's activity.

Around 20 to 25% of pharmaceutical drugs contain fluorine in their structure, the most famous being Prozac.⁵³ Although fluorine is the most abundant of halogens on earth only thirteen fluorinated natural products have been isolated out of more than 3000 isolated halogenated natural products⁵⁴ and only one enzyme with the capacity to fluorinate has been isolated: a fluorinase from *Streptomyces cattleya*.⁵⁵ Out of those thirteen fluorinated compounds 8 are ω-fluorinated homologues of long chain fatty acid derivatives found as co-metabolites in the seeds of the same plant.⁵⁴ Only six original fluorinated natural compounds have been isolated without the eight homologues (**Figure 1.16**).⁵⁴

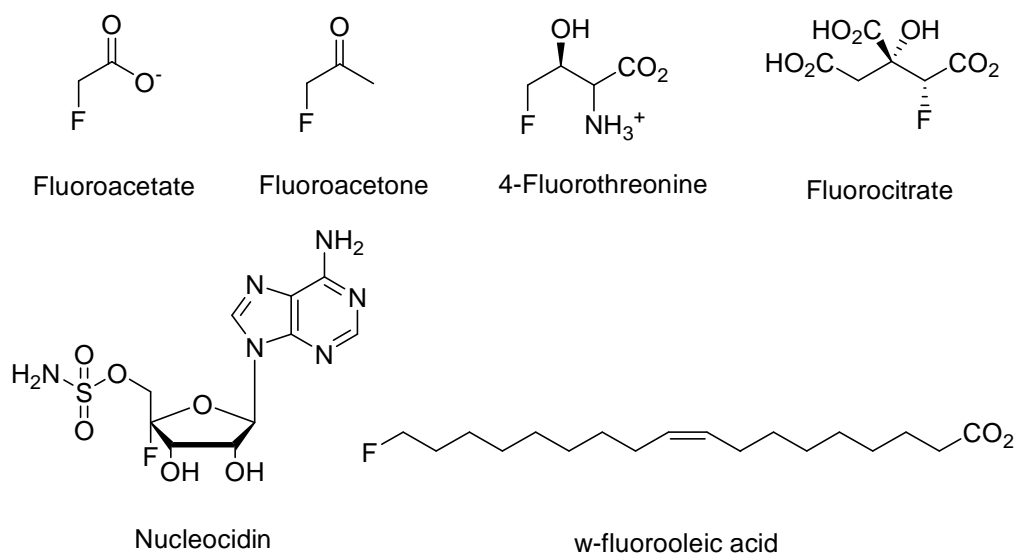
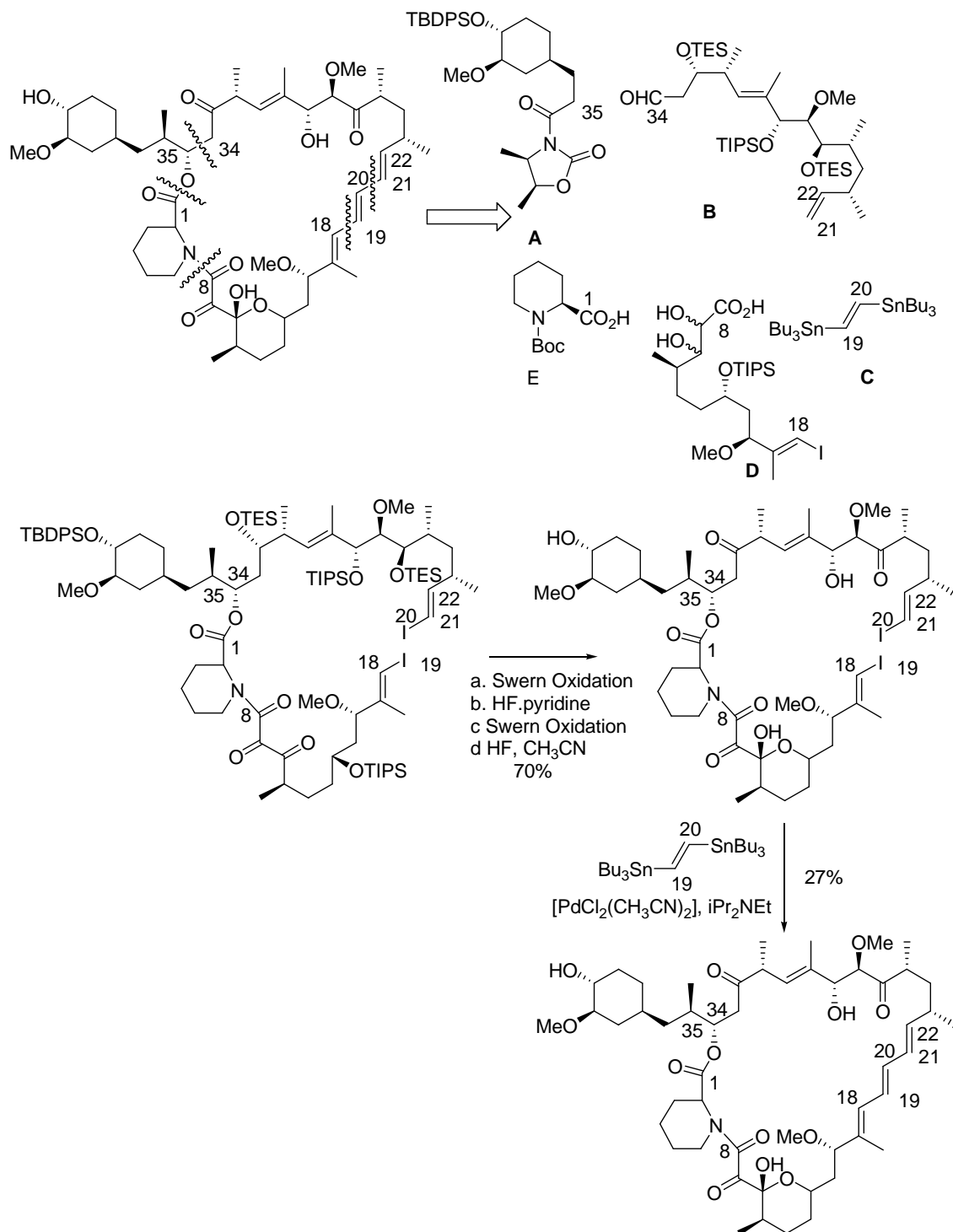


Figure 1.16: Main fluorinated natural products.

The hydroxyl group of rapamycin on carbon 40 could be substituted with a fluorine, but the fluorine can also be placed in other positions around the ring creating a range of fluorinated rapamycins that could be tested for activity and could result in a better understanding of its mechanism.

There are potentially four routes available through which to access fluorinated analogues of rapamycin. The first one is to fluorinate rapamycin chemically. Rapamycin is fairly acid and base labile and attempts toward direct fluorination have been reported as unsuccessful. The second solution is total synthesis. Five total syntheses of rapamycin have been published so far: Nicolaou's,⁵⁶ Schreiber's,⁵⁷ Danyshefsky's,⁵⁸ Smith's⁵⁹ and more recently Ley's.⁶⁰ Nicolaou was the first to successfully synthesise rapamycin. His retrosynthetic analysis of rapamycin consists of assembling five main

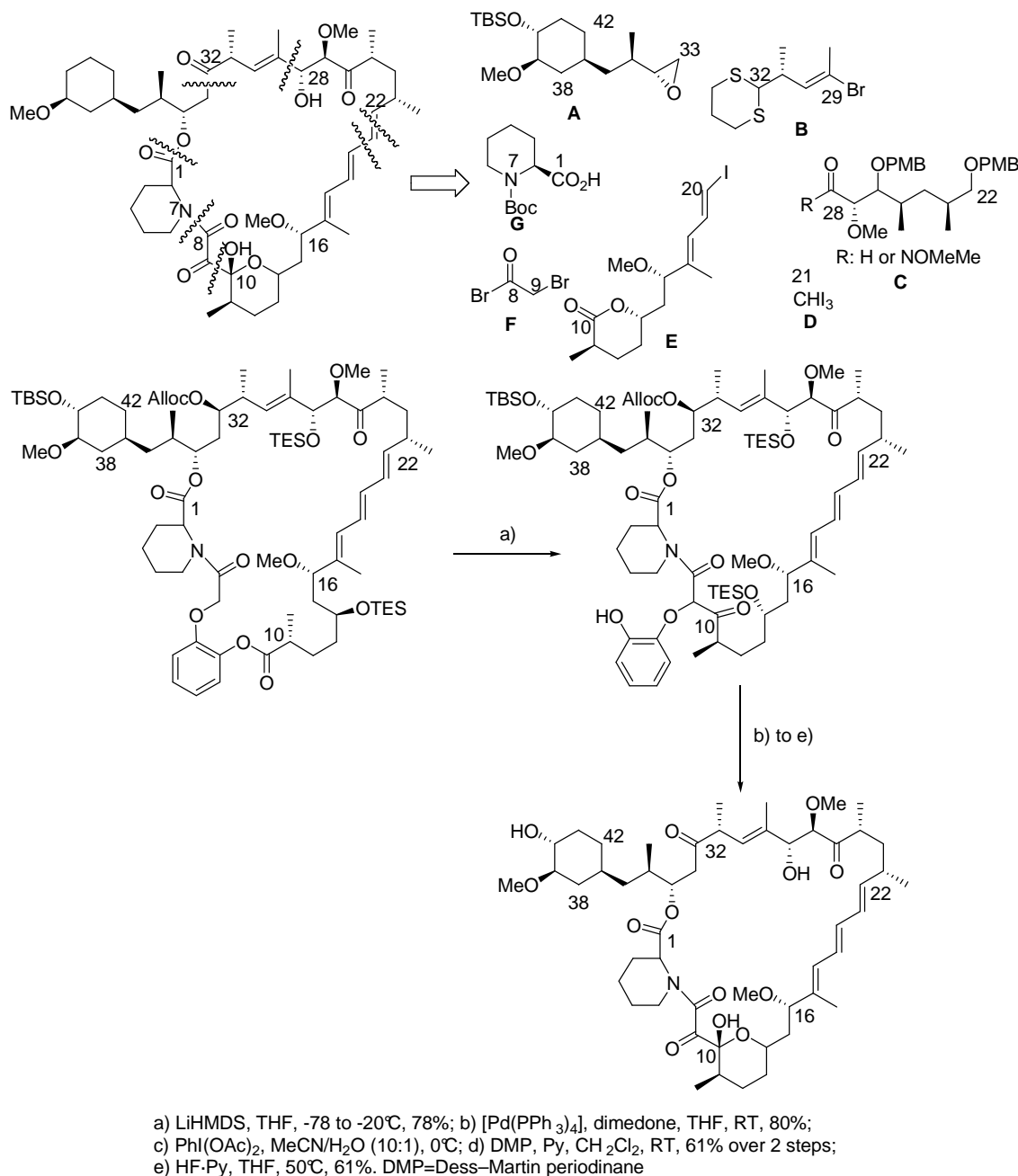
blocks (**Scheme 1.18**). Block A and B were first assembled then E, then D and finally C (**Scheme 1.18**).



Scheme 1.18: Nicolaou's Retrosynthetic analysis of rapamycin and the final steps of his synthesis.⁶¹

The last 2 steps of Nicolaou's synthesis (**Scheme 1.18**) include first a deprotection step followed by a Stille reaction integrating block C ring closing the molecule obtaining

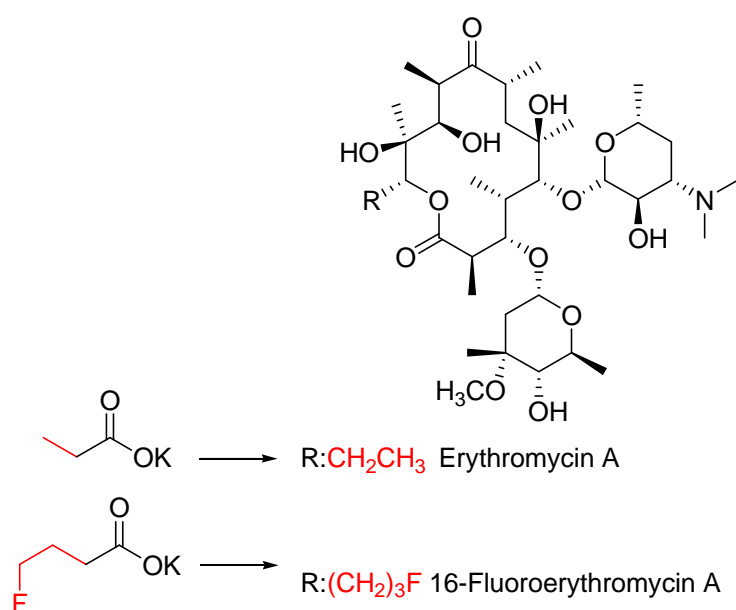
rapamycin. Nicolaou's synthesis is over 40 steps long. Steven Ley's synthesis is the most recent total synthesis of rapamycin to have been completed and is less linear (**Scheme 19**). Ley's reported retrosynthesis below contains 7 fragments. Fragment B and C were first assembled, then A was added, then G, then D, then E and finally F at the same time as a catechol moiety is introduced.



Scheme 19: Ley's retrosynthesis of rapamycin and final steps of his synthesis.⁶⁰

In the final steps of Ley's synthesis the introduced catechol group is used to achieve a remarkable and original ring-closure. The catechol once introduced, formed a macrocycle through both his alcohol groups linking respectively carbon 9 and 10 (**Scheme 19**). Then using a base C9 is deprotonated and attacks C10 ring closing the

main frame of the rapamycin macrocycle expelling the catechol out of the macrocycle. Although very interesting synthetic methodology has been developed, the syntheses are very complex and fairly long: both Ley's and Nicolaou's total synthesis are over 40 steps long and would have to be modified in order to introduce fluorine. The third method would be to introduce fluorinase enzymes into the biosynthesis of rapamycin specifically to introduce fluorine in the wanted position, but this challenge is not realistic. Only one fluorinase enzyme is available and has a narrow substrate specificity for *S*-adenosyl methionine and its close analogues.⁵⁴ The final method, the one used in this project, is mutasynthesis. This is the feeding of a range of fluorinated starter units to a modified *Streptomyces hygroscopicus* lacking the *rapK* gene responsible for the production of the shikimic acid derivative starter unit. The fluorinated starter units will then not be in competition with the natural starter units and can have a better incorporation. The incorporation rates of the starter units will give us some indications on the rules of incorporation of *S. hygroscopicus*. The Goss group had previously produced a fluorinated analogue of erythromycin using precursor directed biosynthesis feeding potassium 4-fluorobutyrate to a modified *Saccharopolyspora erythraea* ERMD1 strain (**Scheme 1.20**).⁶²



Scheme 1.20: Production of fluorinated erythromycin by Goss and coworkers using precursor directed biosynthesis.

A range of alternative fluorinated starter units analogous to the previous incorporated 6 membered rings will be synthesised and fed to *S. hygroscopicus* MG-210.

The first series of starter units targeted will be mono fluorinated cyclohexanoic acids or esters and possibly difluorinated cyclohexanoic acids or esters. Fluoro cyclohexenoic

acid or esters could also be fed. Finally fluorohydrin cyclohexanoic acids or esters will be targeted by synthesis and fed to *S. hygroscopicus* MG-210 in order to produce for the first time a range of fluorinated prerapamycins (**Figure 1.17**).

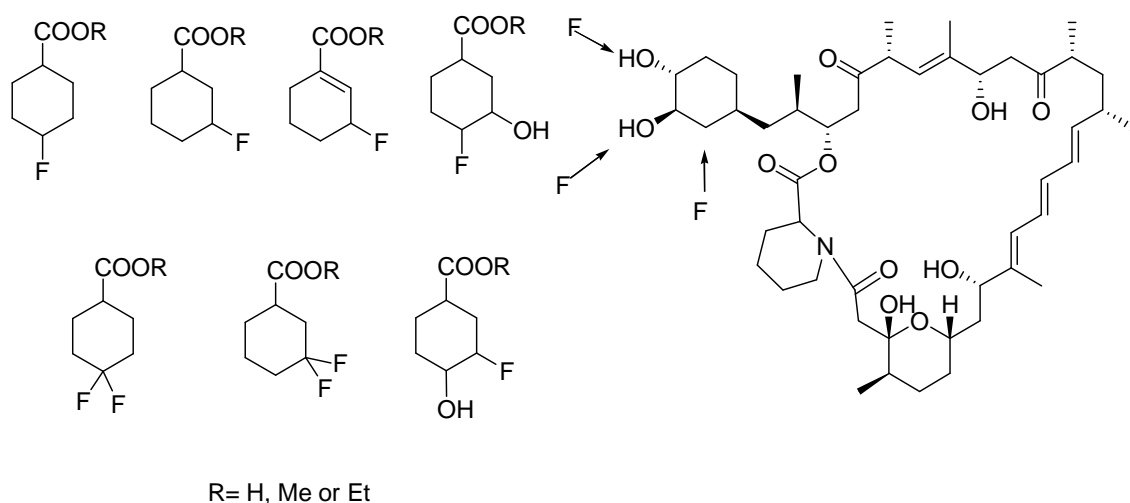


Figure 1.17: Fluorinated compounds targeted for feeding to *Streptomyces hygroscopicus* and rapamycin fluorinated analogues

In the fluorohydrins, in particular, the stereochemistry of the starter units could be modified to create a bigger range of fluorinated prerapamycins and to understand the stereochemical criteria necessary for incorporation.

Once prerapamycins have been produced, *Biotica Technology Ltd* can then produce fluorinated rapamycin using the same starter unit and a modified *S. hygroscopicus* lacking only the gene *rapK*. The fluorinated rapamycin analogues's binding can then be tested with FKPB12 and FRAP in order to have a better understanding of the mechanism of action of rapamycin and possibly to produce a fluorinated rapamycin with enhanced activity.

II- RESULTS AND DISCUSSION CHAPTER

All compounds were synthesised as racemic mixtures (accept for the molecules with a plane of symmetry).

1 SYNTHESIS TOWARD MONOFLUORINATED AND DIFLUORINATED CYCLOHEXANECARBOXYLIC ACIDS AND 3-FLUOROCYCLOHEXENECARBOXYLIC ACID.

Previous feeding experiments carried out by *Biotica Technology Ltd*⁵² demonstrated that 4-hydroxycyclohexanecarboxylic acid **1.94**, 3-hydroxycyclohexanecarboxylic acid **1.95** and 3-hydroxycyclohex-1-enecarboxylic acid **1.97** had been incorporated by *S. hygroscopicus* MG-210, and produced prerapamycin analogues.⁵² The hydroxyl group will be substituted by fluorine. Monofluorinated and difluorinated analogues will be targeted (**Figure 2.1**).

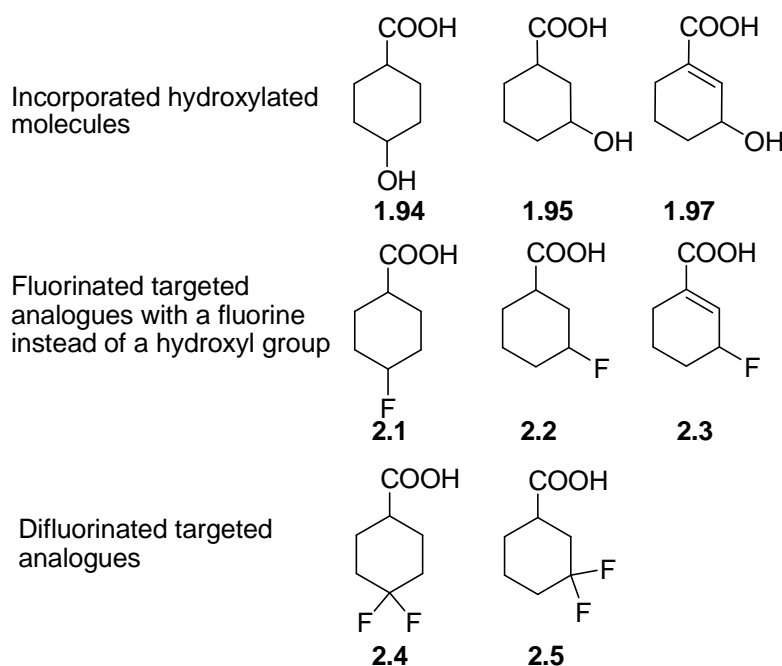
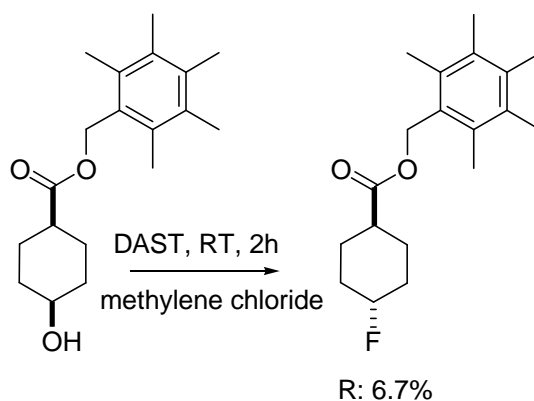


Figure 2.1: Previously incorporated starter units and targeted fluorinated analogues.

4-Fluorocyclohexanecarboxylic acid **2.1**, 3-fluorocyclohexanecarboxylic acid **2.2**, 3-fluorocyclohexenecarboxylic acid **2.3**, 4,4-difluorocyclohexanecarboxylic acid **2.4** and 3,3-difluorocyclohexanecarboxylic acid **2.5** were first targeted.

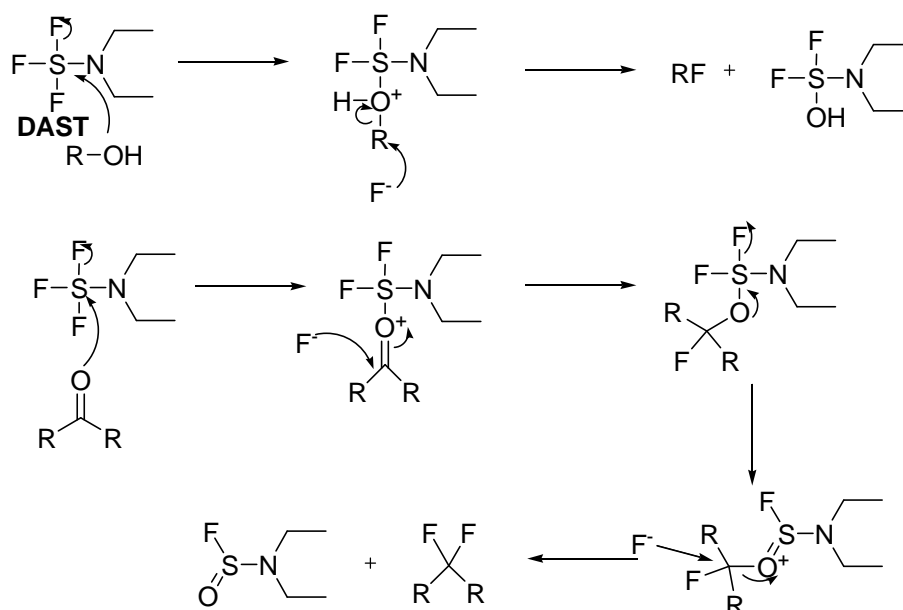
Previously the Goss group, in order to produce all 3 monofluorinated cyclohexanecarboxylic acids had tried to reduce the double bonds of the equivalent fluorinated aromatics,^{52, 63} using hydrogenation at high pressure, using a range of metal catalysts, but it resulted in an inseparable mixture with over 90% of product with a cleaved fluorine group.

A literature synthesis of 4-fluorocyclohexanecarboxylic acid **2.1** already existed prior to the start of the project.⁶⁴ The fluorinating step was clearly the limiting factor of the synthesis. The displacement of the hydroxyl group by fluorine using diethyl amino sulphur trifluoride (DAST) only resulted in a 6.7% yield (**Scheme 2.1**).⁶⁴



Scheme 2.1: Previous fluorination step by LANG and coworkers leading to the synthesis of 4-fluorocyclohexanecarboxylic acid **2.1**.⁶⁴

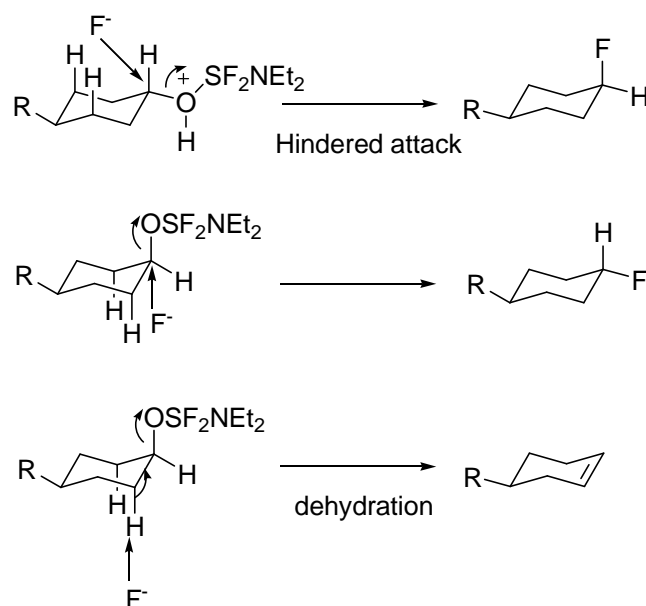
DAST is usually one of the most efficient reagents in order to substitute a hydroxyl group with fluorine. DAST's sulphur is very electrophilic because of the three fluorines bonded to it and will readily be attacked by nucleophiles such as alcohols or even ketones that are not good nucleophiles. The reaction has to be carried out at a higher temperature in order to fluorinate ketones. Once attacked by the alcohol, DAST will release a fluorine anion (**Scheme 2.2**). Ketones are usually difluorinated; DAST releases two fluorine anions in that case (**Scheme 2.2**).



Scheme 2.2: Fluorination mechanism of alcohols and ketones with DAST.

Once the fluorine is released it can then substitute the hydroxyl group through an S_N2 type of substitution, inverting the configuration of the molecule in case the alcohol is on a stereocenter. DAST due to its high reactivity has the disadvantage of being unstable to heat and explosive in contact with water.

This poor yield of 6.7% during the fluorination with highly reactive DAST can be explained by Middleton's hypothesis.⁶⁵ The generation of fluorocyclohexanecarboxylic acids is not trivial; if the hydroxyl group is in an axial position the approach of the fluorine in an S_N2 type of substitution will be difficult due to steric hindrance (**Scheme 2.3**). If the hydroxyl group is in an equatorial position, then the hydroxyl group can be substituted by fluorine, but the fluorine anion can also act as a base leading to elimination of the previous hydroxyl group (**Scheme 2.3**).



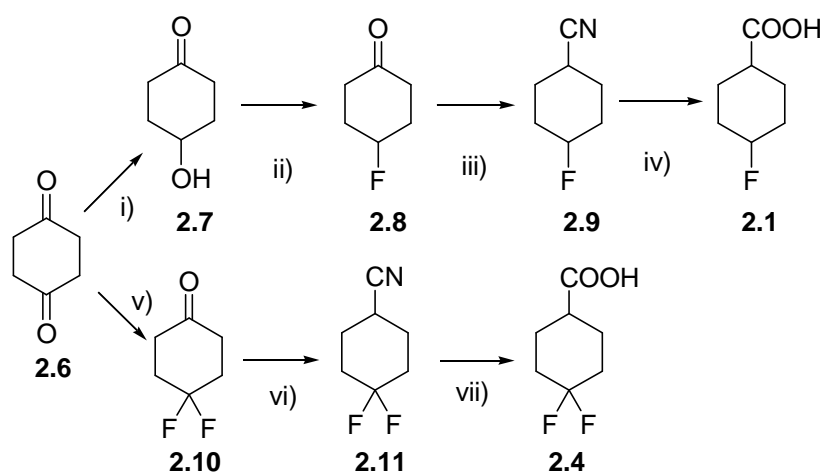
Scheme 2.3: Explanation for poor yields in the fluorination of substituted cyclohexanols, adapted from Mange and Middleton.⁶⁵

We therefore set out to develop an alternative approach, to access these compounds, by fluorinating a flattened intermediate compound containing one or more sp^2 carbons in its 6-membered ring, therefore limiting the steric hindrance and dehydration. The ring could contain a ketone or a double bond to be reduced later. A literature procedure towards the synthesis of 3-fluoro-cyclohex-1-enecarboxylic acid **2.3** already existed in the literature with a reported fluorination step with a 69% yield.⁶⁶ The double bond could be possibly reduced and 3-fluorocyclohexanecarboxylic acid **2.2** could be accessed. In order to access 4-fluorocyclohexanecarboxylic acid **2.1** it was decided to fluorinate 4-hydroxycyclohexanone **2.8** and to try and transform the ketone into a carboxylic acid. The synthesis of 4-fluorocyclohexanecarboxylic acid **2.1** was first investigated (**Scheme 2.4**).

1.1 Synthetic Route Toward 4-Fluorocyclohexanecarboxylic Acid **10** and 4,4-Difluorocyclohexanecarboxylic Acid **14**.

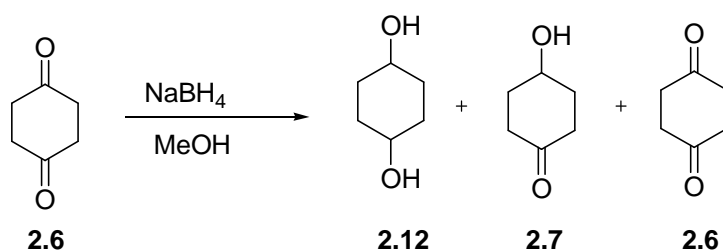
The route investigated could allow us to access the mixture of *cis* and *trans* 4-fluorocyclohexanecarboxylic acid **2.1** and 4,4-difluorocyclohexanecarboxylic acid **2.4** using cyclohexane-1,4-dione **2.6** as a cheap, stable, commercially available starting material (**Scheme 2.4**).

Diketone **2.6** would be monoreduced using sodium borohydride into 4-hydroxycyclohexanone **2.7**, then fluorinated using DAST or HF (complexed to pyridine or triethylamine) to give 4-fluorocyclohexanone **2.8**, followed by the transformation of ketone **2.8** using tosyl methyl isocyanide into 4-fluorocyclohexanecarbonitrile **2.9**, and finally hydrolysing the cyano group to give 4-fluorocyclohexanecarboxylic acid **2.1**. In a similar fashion diketone **2.6** could be difluorinated using DAST to give 4,4-difluorocyclohexanone **2.10**, then transformed using tosyl methyl isocyanide into 4,4-difluorocyclohexanecarbonitrile **2.11** and finally hydrolysed into 4,4-difluorocyclohexanecarboxylic acid **2.4**.



Scheme 2.4: Second potential synthetic route toward 4-fluorocyclohexanecarboxylic acid **2.1** and 4,4-difluorocyclohexanecarboxylic acid **2.4**

1.1.1 The reduction step (literature procedure).⁶⁷



Scheme 2.5: Reduction of the diketone **2.6** using NaBH_4

Only one literature procedure was available in order to reduce cyclohexane-1,4-dione **2.6** in 4-hydroxycyclohexanone **2.7**.⁶⁷ The reported procedure was vague and claimed a 60% yield. When the procedure was repeated it only resulted in a 24% yield.

This is a non-selective reduction and results in a mixture of starting material **2.6**, monoreduced compound **2.7**, and the di-reduced diol **2.12** (**Scheme 2.5**). The formation

of all 3 compounds was easily followed by TLC (in a 100% ethyl acetate R_f **2.12**: 0.15, R_f **2.7**: 0.26 R_f **2.6**: 0.50). In order to optimise the yield toward the mono reduced compound this reaction was carried out a number of times varying the number of equivalents of NaBH₄, the reaction temperature, the time taken for the addition of NaBH₄, and the reaction time (**Table 2.1**).⁶⁷

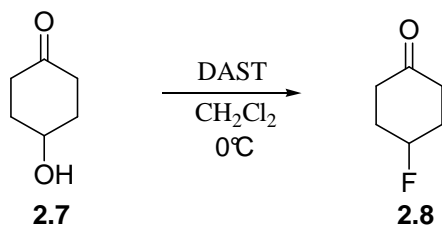
Exp. n°	N° of equivalents of NaBH ₄	T (°C) RANGE	Time (h)	Addition time of NaBH ₄	Isolated yield (%)
1	1/4	-16	2.5	1s	3
2	1/4	-16 to 25	10 + 10	1s	17 ^a
3	1/4	-16 to 25	10 + 10	1s	11 ^a
4	1/4	-10	2.5	5 min.	24
5	1/3	-10	2.5	5 min.	36
6	4/10	-10	2.5	5 min.	27
7	1/3	-10	2	5 min.	21
8	1/3	-10	3	5 min.	32

^a impurities observed by ¹H NMR

Table 2.1: Summary of the main reduction attempts on diketone **2.6** with NaBH₄

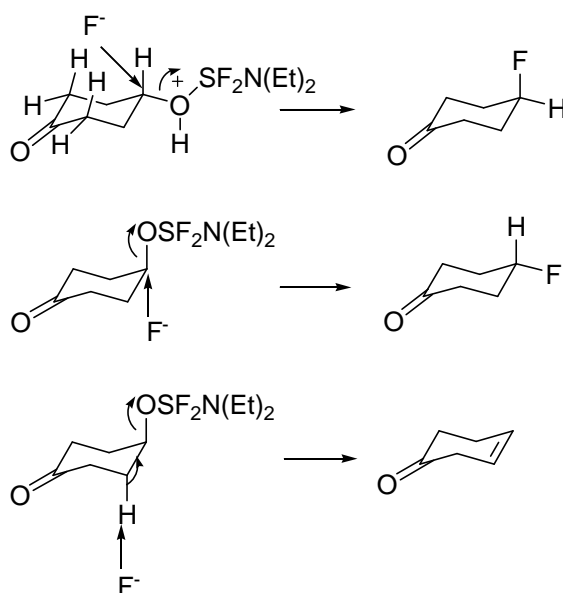
The key factor seems to be the temperature control. At -16°C hardly any reduction occurs. When the temperature of the reaction is allowed to rise to room temperature, a high production of diol **2.12** is observed and some new impurities with similar R_f to the monodiol **2.7** appear. The yield is optimal at around -10°C. NaBH₄ must be added very slowly in order to control the reaction temperature, if added too fast the temperature goes up and the selectivity of the reaction drops, and more diol **2.12** is produced. The reaction was optimised using 1/3 of an equivalent of NaBH₄ (addition of more NaBH₄ resulted in higher amounts of diol **2.12**) and a reaction time of 2.5 hours. The optimised yield in our hands was 36%.

1.1.2 The monofluorination step.^{68, 69}



Scheme 2.6: Fluorination of the alcohol **2.7** using DAST

A range of three fluorinating agents were tested in order to fluorinate **2.7**: HF pyridine,⁷⁰ HF triethyl amine⁷¹ and diethyl amino sulphur trifluoride (DAST).⁶⁹ Only DAST resulted in fluorination of **2.7**, due to its high reactivity, but elimination product was still detected (**Scheme 2.6 and 2.7**).^{68, 69}



Scheme 2.7: Fluorination and Elimination mechanism.⁶⁵

In order to optimise the yield toward the mono fluorinated compound the reaction was carried out a number of times varying the concentration of DAST, the concentration of starting material and the reaction time (**Table 2.2**).

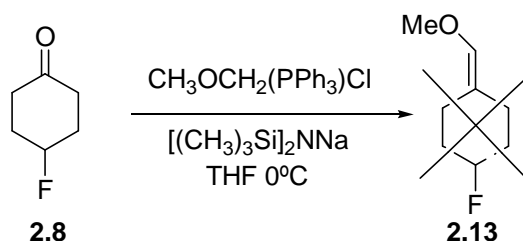
Experiment number	[2.7] (mmolL ⁻¹)	[DAST] (mmolL ⁻¹)	Reaction time at 0°C (min.)	Isolated yield (%)
1	187	7572 (neat)	30	1
2	21.9	132	60	18
3	19.7	132	60	21
4	21.4	86.2	60	20

Table 2.2: Summary of the most representative fluorination attempts on compound **2.7** using DAST.

In experiment 1, neat DAST was added to a relatively concentrated solution of starting material in DCM and was stirred at 0°C until completion for 30 minutes. After workup, this gave a mixture of the fluorinated compound (minor), elimination product (major) and various other impurities. After purification only 1% of compound was isolated. In experiment 2, 3 and 4 the reaction went to completion and the desired product was the major one. In these experiments much less elimination compound was observed on TLC. In all 4 experiments the product was purified by flash chromatography on silica gel using a 90:10 Petrol/ Ethyl acetate mixture. An improvement in yield was obtained by dilution of the DAST;⁶⁹ this is the key factor in reducing levels of unwanted side products. Dilution of DAST prior to addition allows the reaction temperature to rise gently, maintaining the selectivity of the fluorination and reducing the elimination. A yield of 21% for the fluorination step was an improvement on the previously reported 6.7% yield for fluorination,⁶⁴ probably linked to the fact that 4-hydroxycyclohexanedione **2.7** is slightly less sterically hindered than a cyclohexane ring, due to the flattening of the ring by the presence of the sp² carbon of the ketone. The hydrogens on the carbons adjacent to the ketone are pseudo equatorial and pseudo axial this allows a slightly less hindered approach for the fluorine.

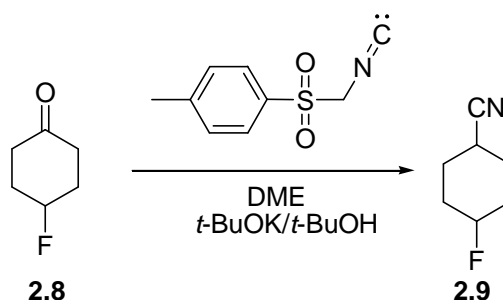
In order to access the final compound the ketone would have to be transformed into an acid. This could be achieved over two steps. *Biotica Technology Ltd* had attempted Wittig reactions on similar systems previously but with little success. Nevertheless the Wittig reaction was attempted for this particular molecule adding (methoxymethyl)triphenylphosphonium chloride and sodium bis(trimethylsilyl)amide in THF at 0°C to the compound, but no reaction was observed (**Scheme 2.8**).⁷² The next

method investigated was a Knoevenagel type reaction transforming the ketone **2.8** into the nitrile **2.9**.



Scheme 2.8: Failed Wittig reaction

1.1.3 Knoevenagel type reaction converting the ketone group into a nitrile of the monofluorinated compound.⁷³



Scheme 2.9: Knoevenagel type reaction⁷³

Experiment number	Crude Yield (%)	Purification method	Degradation during purification	Isolated yield after purification (%)
1	87	Silica Column	Yes	0
2	90	Basified silica column	Yes	9
3	92	Filtration in ether	No	88 ^a

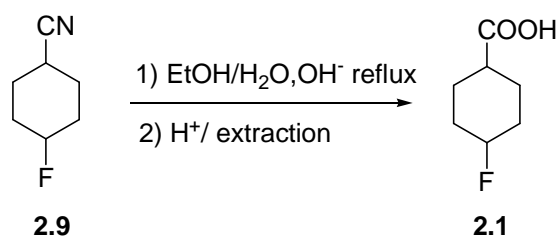
^a trace impurities ¹H NMR

Table 2.3: Summary of the different Knoevenagel type reactions and purification attempts.

The reaction was successful and went to completion but the product degraded during purification by flash chromatography on silica (**Table 2.3** Experiment 1). The purification by flash chromatography was next carried out on silica previously neutralised with a little triethylamine in the solvent mixture. This was also unsuccessful as most of the compound degraded (**Table 2.3** Experiment 2). The TLC indicated that the crude mixture had one main impurity and the ¹H NMR spectrum of the crude mixture indicated it was aromatic. By dissolving the mixture in various solvents it was

observed that most of the impurity could be removed dissolving the crude compound in cold diethyl ether in which only the desired compound was soluble. The impurity was removed by filtration and the organic solution was evaporated to yield the almost pure compound as a mixture of *cis* and *trans* compound with a 45 : 55 ratio as suggested by the proton ratio of the hydrogen adjacent to the carboxylic acid from the ^1H NMR spectrum (**Table 2.3** Experiment 3). Finally, the nitrile group was hydrolysed to yield the 4-fluorocyclohexanecarboxylic acids **2.1**.

1.1.4 Hydrolysis of the nitrile into the acid.⁷⁴



Scheme 2.10: Hydrolysis of the nitrile group into the carboxylic acid.

This reaction was carried out five times in order to optimise the yield. In each case all the starting material was consumed. The factors examined were the quality of the starting material, the reaction time, the temperature, and the pH of the solution during extraction (**Table 2.4**).

Exp. Number	T of oil bath in (°C)	pH	Time (h)	Starting material in freezer	Yield after purification (%)	Pure mixture of both acids
1	96	0.5	1.5	2 days crude	0	
2	88	1.5	2	2 days crude	35	No
3	84	2	2.5	0 days crude	42	No
4	85	2	3.5	9 days crude	0	No
5	82	1.5	4	4 days pure	60	Yes

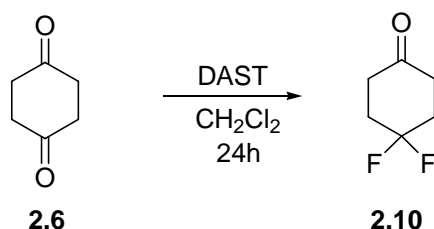
Table 2.4: Summary of the different hydrolysis reaction.

Initially the starting material was not purified therefore the reaction was carried out on the crude starting material. It was believed that the impurities could be removed by extraction of the basic solution prior to the acidification of the solution in order to extract the acid. The longer the impure starting material was kept in the freezer before being reacted the worse the reaction yield. The starting material as an impure mixture

would start degrading in the freezer. It was also observed that no product was detected in experiment 1 compared to 2. The absence of product in experiment 1 could be explained by degradation possibly due to the higher temperature of the reaction or the very low pH of the solution when acidified prior to extraction. The final product using the crude starting material contained impurities. The product could be purified using an exchange resin but due to the small quantity involved and the success of a partial purification of the starting material the experiment was repeated on the purified mixture. In experiment 5, the starting material was 4 days old but had been previously purified by filtration (with cold ether) as previously described and the ^1H NMR spectrum on the day of the reaction showed that the starting material had not degraded. Experiment 5 gave a pure mixture of both targeted isomers in a 60% yield. In order to optimise the reaction, the starting material should be purified, the oil bath temperature should be controlled, and so should the pH of the solution before extraction. Compound **2.1** was fed as the *cis* and *trans* mixture to *S. hygrosopicus* by *Biotica Technology Ltd.*

1.1.5 Difluorination of the ketone ⁷⁵

Cyclohexane-1,4-dione **2.11** was also used as starting material in the first step of the synthesis toward 4,4-difluorocyclohexanecarboxylic acid **2.4**. DAST as previously seen in **Scheme 2.2** can also difluorinate ketones⁶⁸ and was chosen as fluorinating agent in this synthesis. In the literature cyclohexanone had been difluorinated in that manner in DCM at room temperature by Dolbier and coworkers,⁷⁵ but no yield was mentioned. Similar conditions were used in this step. In order to optimise the reaction, the reaction was repeated varying the number of DAST equivalents, the reaction temperature, the concentration of the reactants, and the purification method (**Table 2.5**).



Scheme 2.11: Difluorination of the diketone **2.6** using DAST.

Exp. N°	[2.6] (molL ⁻¹)	N° of equiv. of DAST	T. (°C)	Presence of [2.10] after work up	Separation	Yield (%)
1	0.17	1	20	Yes	Silica	0
2	0.45	0.89	20	Yes	Basified Silica	10
3	0.45	1	20	Yes	Filtration	6
4	0.11	1	45	No	None	0

Table 2.5: Summary of the different fluorination attempts.

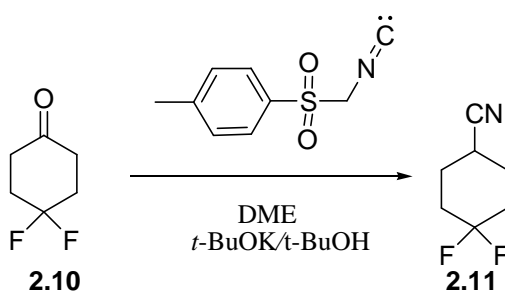
The reaction was followed by TLC and GCMS. It was first carried out at 0°C and 10°C but no product formed. It was left to reach room temperature and was stirred under anhydrous conditions for 24 hours. After 24 hours the degradation of the product would increase faster than its production. Fluorination would not occur at lower than room temperature because of the lower nucleophilic potential of the oxygen of the ketone compared to the previous alcohol.

The crude product was yielded as a mixture of cyclohexane-1,4-dione **2.6** and 4,4-difluorocyclohexanone **2.10**, no tetrafluorinated compound was detected but other unidentified impurities were also present. At these higher temperatures DAST degrades, and the unstable difluorinated compound generated can also start to break down.

During purification, the product degraded on silica. On neutralised silica (neutralised with triethylamine) the compound was partially purified, but some new degradation spots appeared on the TLC. A partial purification was achieved by dissolving the crude mixture in cold hexane (some impurities would not dissolve). The partially purified product degraded after 10 days in the freezer under N₂. The purification by filtration had the advantage of being very fast and allowed us to react the unstable product in the next step. The rest of the synthesis should be carried out in a short period of time because of the speed of product degradation. The compound was unstable at room temperature and no carbon NMR spectrum was obtained (product degraded in CDCl₃).

1.1.6 Knoevenagel type reaction on the difluorinated compound.

In order to transform the ketone into the carboxylic acid the same method as previously used was applied.⁷³



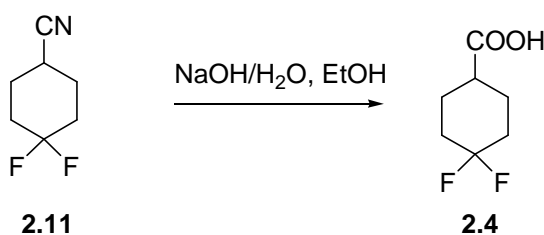
Scheme 2.12: Transformation of the ketone **2.10** into cyano product **2.11**

Experiment number	[28] mmolL ⁻¹	Starting material purified by	Number of equivalents	Time (h)	Product detected
1	28	column	1.1	4.5	No
2	52	filtration	2.5	2.5	Yes
3	69	filtration	2.2	12	Yes

Table 2.6: Summary of the different Knoevenagel type reactions.

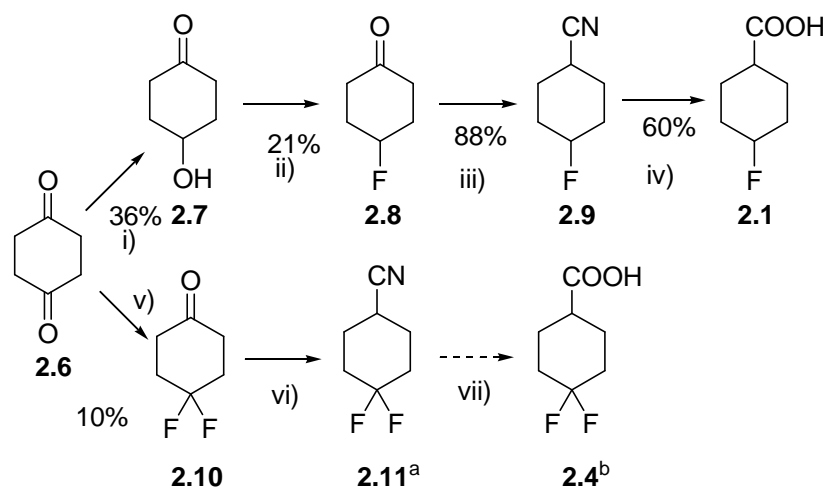
In experiment 2 and 3 the starting material **2.10** was reacted straight after being worked up and purified by filtration (**Table 2.6**). The reaction was followed by GCMS and TLC. A mixture of 4,4-difluorocyclohexanecarbonitrile **2.11**, an aromatic compound, and other unidentified impurities were obtained (**Table 2.6**). Purification with column chromatography on silica and neutralised silica resulted in total degradation of the product. Some impurities were removed, as described previously, dissolving the mixture in cold diethyl ether and filtering out the impurities. The product was still impure, but most of the aromatic impurity had been removed.

1.1.7 Hydrolysis attempt of the nitrile group in the acid of the difluorinated compound.



Scheme 2.13: Hydrolysis of the cyano group.

The impure compound was hydrolysed in similar conditions as previously used for the monofluorinated compound,⁷⁴ but only traces of hydrolysed compound were detected by mass spectrometry. The final difluorinated acid was purchased by *Biotica Technology Ltd* (10 mg) from *Matrix Scientific*. No incorporation was observed in *S. hygroscopicus* MG-210. The feeding results led to abandoning the synthesis.



i) NaBH₄, MeOH, -10°C, 2,5h ii) DAST, DCM, 0°C, 1h iii) TO SMIC, DME, *t*-BuOK, *t*-BuOH, 25°C, 3h iv) EtOH, NaOH/ H₂O v) DAST, DCM, 25°C, 24h vi) TOSMIC, DME, 25°C, 3h vii) EtOH, NaOH/ H₂O
^a impure compound ^b traces detected

Scheme 2.14: Summary of the synthesis of 4-fluorocyclohexanecarboxylic acid **2.1** and attempted synthesis of 4,4-difluorocyclohexanecarboxylic acid **2.4**.

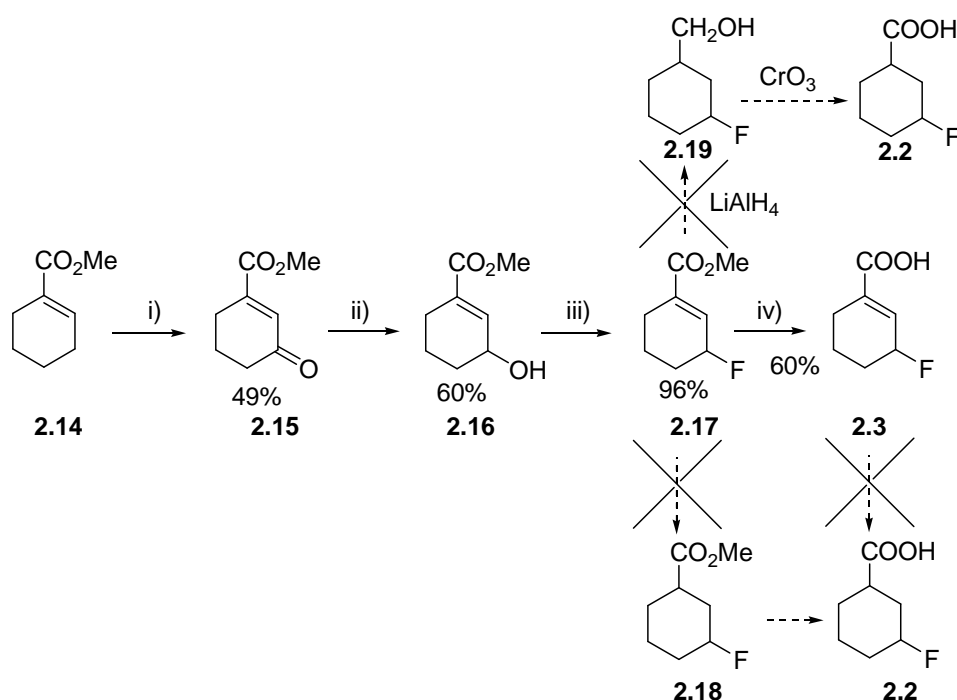
4-Fluorocyclohexanecarboxylic acid was successfully synthesised in 4 steps (Scheme 2.14), with an improved fluorination step compared to the previous literature procedure. Another method to access compound **2.1** is to synthesise 3-fluoro-cyclohex-1-enecarboxylic acid **2.3**, and to attempt a reduction of the double bond.

1.2 Synthesis of 3-Fluoro-cyclohex-1-enecarboxylic Acid and Attempt to Reduce its Double Bond.

1.2.1 Synthesis of 3-fluoro-cyclohex-1-enecarboxylic acid.⁶⁶

In order to synthesise 3-fluoro-cyclohex-1-enecarboxylic acid **2.3** all four steps of the synthesis were repeated from a literature procedure from Bridge, O'Hagan and coworkers.⁶⁶ The fluorination step using DAST in DCM has a reported yield of 69% yield.⁶⁶ Such a high yield compared to the previous fluorinations with DAST can be explained by the strongly flattened cyclohexene ring compared to a cyclohexane ring.

The cyclohexene ring contains 2 sp^2 carbons. There is relatively little steric hindrance for the fluorine to approach.



i) CrO_3 , Benzene, acetic acid and acetic anhydride, RT, 45 min ii) CeCl_3 , NaBH_4 , MeOH, RT, 15 min
 iii) DAST, DCM, 0°C , 3.5 h iv) NaOH, EtOH, RT, 3h.

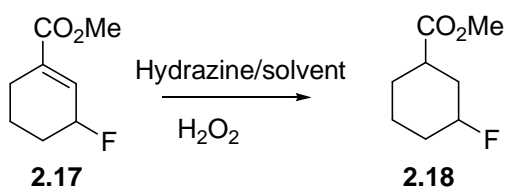
Scheme 2.15: Synthetic route towards 3-fluorocyclohex-1-enecarboxylic and 3-fluorocyclohex-1-enecarboxylic acid.

Methyl cyclohex-1-enecarboxylate **2.14** was oxidised with CrO_3 to methyl 3-oxocyclohex-1-enecarboxylate **2.15** in a 49% yield.⁷⁶ This was then reduced to methyl 3-hydroxycyclohex-1-enecarboxylate **2.16** in a 60% yield,⁷⁷ followed by fluorination using DAST in methyl 3-fluorocyclohex-1-enecarboxylate **2.17** with a 96% yield.⁶⁶ Finally the ester was hydrolysed to 3-fluoro-cyclohex-1-enecarboxylic acid **2.3**, but the acid was slightly impure (purification with an exchange resin may improve this).⁶⁶ The ester and the acid were fed by *Biotica Technology Ltd* to *S. hygroscopicus* MG-210.⁵²

1.2.2 Double bond reduction attempt.

The reduction of the double bond was then attempted on the ester. The Goss group had previously tried hydrogenation using various catalysts Ru/C, Pd/C, Ru/Al, Ru/C, but these conditions led to no reduction or reduction with loss of the fluorine.

Hydrazine was then tested in order to reduce the double bond, forming a diimide intermediate.⁷⁸ Traces of compound were detected by GCMS and optimisation of the reaction was attempted, changing various conditions: reaction time, temperature, solvents, number of equivalents of hydrazine, concentration of the starting material, adding various catalysts Cu(II) (CuSO₄), acetic acid associated with sodium periodate (**Table 2.7**).⁷⁸



Scheme 2.16: Reduction of the double bond using hydrazine⁷⁸

Exp. n°	Time (h)	NH ₂ NH ₂ (equiv.)	[2.17] (mmolL ⁻¹)	Solvent	CuSO ₄ solution	CH ₃ CO ₂ H and NaIO ₄	H ₂ O ₂ addition Time	T (°C)	Product detected by GCMS
1	4	22	46.0	EtOH	No	No	5 min	0	Yes
2	4	100	24.4	MeOH	Yes	Yes	5 min	25	No
3	4	22	232	EtOH	Yes	No	10 s	25	No
4	4	22	244	EtOH	No	No	10 s	0	No
5	4	25	45	MeOH	No	No	5 min	0	No
6	16	22	47	EtOH	Yes	No	1.5 h	0	Yes
7	80	22	88	EtOH	Yes	No	1.5 h	0	Yes

Table 2.7: Summary of the different reduction attempts using hydrazine

In experiment 1, 6 and 7 the main product had a mass of 171, and the possible reduced product with a mass M+1 of 161 was detected as the minor product. Experiment 7 according to the GCMS seems to be the most optimised. Ethanol seemed to be the best solvent; the addition of H₂O₂ should be performed slowly and at low temperature, over a long period of time, under nitrogen. The yields were very poor, and not enough product was present to observe it by ¹H NMR, to confirm if it was the reduced product. A nucleophilic attack on the double bond was suspected by hydrazine. In order to avoid this problem azodicarboxylate as a source of diimide was tried next using protic and

aprotic solvents,⁷⁸ but no reduction was observed. All these experiments were repeated on the acid instead of the ester but the reactivity did not change.

Another way to reduce the double bond would be reduction of the double bond and the ester to the alcohol, using an excess of lithium aluminium hydride (LiAlH₄) and then oxidation of the resulting alcohol in the acid. The reduction of the ester with LiAlH₄ to the alcohol was carried out from -10°C to 0°C. At -10°C no reaction occurred, but carried out at 0°C the reaction resulted in the cleavage of the fluorine bond.

As the reduction of the double bond failed a different synthesis was attempted to access 3-fluorocyclohexanecarboxylic acid **2.2** using the same method to the prior synthesis of 4-fluorocyclohexanecarboxylic acid **2.1** using as starting material cyclohexane-1,3-dione.

1.3 3-Fluorocyclohexanecarboxylic Acid Synthesis.

3-Fluorocyclohexanoic acid would be accessed using the same method as previously except that cyclohexane-1,3-dione was first protected before being reduced.⁷⁹ Direct reduction using NaBH₄ did not work because of the acidic protons between both ketones, NaBH₄ acting as a base instead of a reducing agent (**Figure 2.2**).

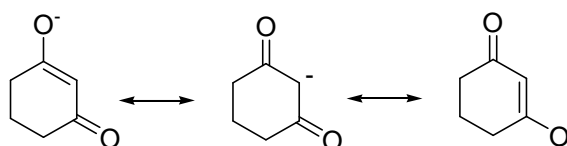
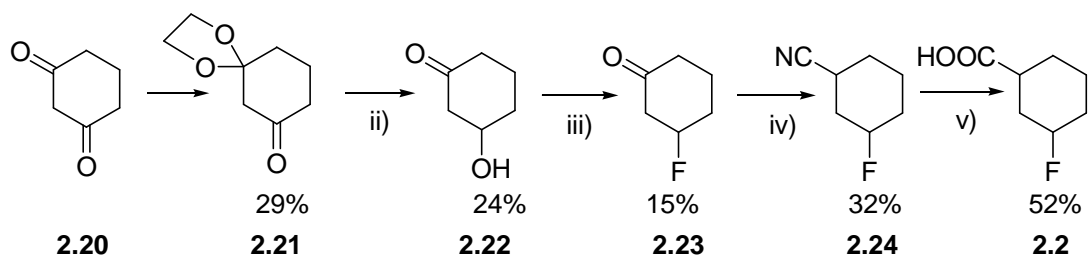


Figure 2.2: Stabilisation of the deprotonated compound.



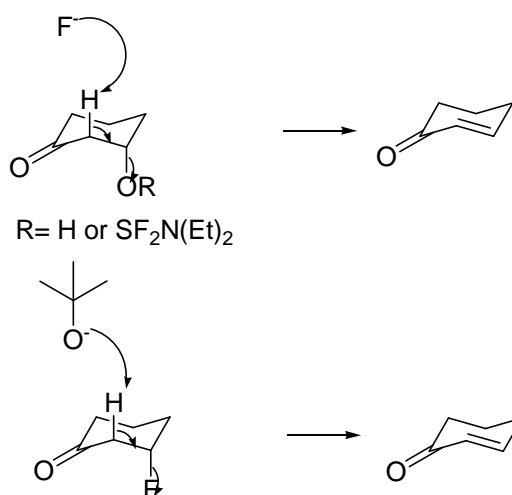
i) (CH₂OH)₂, *p*-TsOH, C₆H₆, reflux, 3.5 h ii) NaBH₄, MeOH, -10°C, 1h then HCl iii) DAST, DCM, 0°C, 4h iv) TOSMIC, DME, *t*-BuOK, *t*-BuOH, 25°C, 1.5h v) EtOH, NaOH/ H₂O, 3h, reflux

Scheme 2.17: Synthesis toward 3-fluorocyclohexanecarboxylic acid.

Diketone **2.20** was protected with ethylene glycol to afford 1,4-dioxaspiro[4.5]decan-7-one **2.21** in a 29% yield.⁷⁹ The ketone was then reduced using sodium borohydride and

the alcohol deprotected with HCl to give 3-hydroxycyclohexanone **2.22** in a 24% yield.⁶⁷ **2.22** was then fluorinated using DAST to give 3-fluorocyclohexanone **2.23** in a 15% yield.⁶⁸ The fluorination was followed by a Knoevenagel type reaction using tosyl methyl isocyanide to transform the ketone **2.23** in a mixture of *cis* and *trans* 3-fluorocyclohexanecarbonitrile **2.24** in a 32% yield.⁷³ Finally the cyano group was hydrolysed to a mixture of *cis* and *trans* 3-fluorocyclohexanecarboxylic acid **2.2** with a 1 : 1 ratio in a 52% yield.⁷⁴

The fluorination step and the Knoevenagel type reaction had lower yields than observed for the previous procedure with the 1, 4 diketone **2.6**. This is probably due to elimination occurring in the fluorination step and during the Knoevenagel type reaction. This is due to the acidic protons adjacent to the ketone and to the carbon linked to the hydroxyl group or the fluorine, facilitating the elimination of the the hydroxyl group or the fluorine (**Scheme 2.18**).



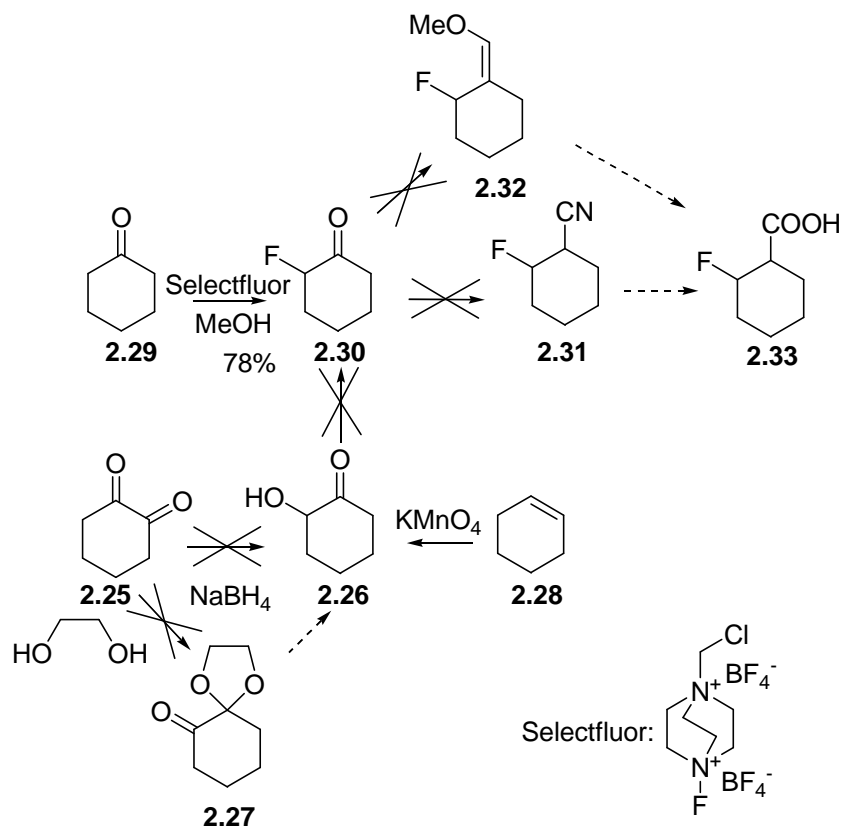
Scheme 2.18: Possible elimination occurring during the synthesis

Similar methods were used to produce 4 and 3 fluorocyclohexanecarboxylic acid. The same method was tested in regard to the synthesis of 2-fluorocyclohexanecarboxylic acid.

1.4 Attempt to synthesise 2-fluorocyclohexanecarboxylic acid.

In order to compare fluorination rates the synthesis of 2-hydroxycyclohexanone **2.26** was first attempted using NaBH₄ on the diketone **2.25** as previously described, but no reduction was observed even at room temperature. A monoprotection of **2.25** was then attempted as previously performed with 3-fluorocyclohexanecarboxylic acid, using ethylene glycol, but no protected product **2.27** was observed. The failure of both reactions must be due to fact that both ketones of **2.25** are conjugated and therefore non

reactive. The mono alcohol **2.26** was finally obtained following a literature procedure resulting from the oxidation of cyclohexene **2.28** using potassium permanganate and copper sulphate in *tert*-butanol in a 21% yield.⁸⁰



Scheme 2.19: Synthesis attempted toward 2-fluorocyclohexanecarboxylic acid **2.33**.

The fluorination of 2-hydroxycyclohexanone **2.26** was next attempted using DAST in similar conditions as previously for 4- and 3-hydroxycyclohexanone, but no fluorination was observed. Although the sterics were more favourable to the approach of the DAST than for 3-hydroxycyclohexanone (15% yield for the fluorination step) and 4-hydroxycyclohexanone (22% yield for the fluorination step; the flatter the 6 membered ring the better the yield of the fluorination as observed with 3-fluorocyclohexanecarboxylic acid: 96% yield for the fluorination step), 2-hydroxycyclohexanone didn't react with DAST. The lack of reactivity of the alcohol as a nucleophile, not attacking the electrophilic sulphur of DAST must be due to the tautomeric equilibrium between the ketone and the hydroxyl (**Figure 2.3**), therefore no reaction is observed. HF pyridine in DCM, neat HF triethylamine were also tested but without any success.

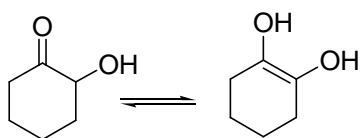


Figure 2.3: Keto-enol tautomerism of 2-hydroxycyclohexanecarboxylic acid **2.26**.

2-Fluorocyclohexanone **2.30** was finally obtained following a literature procedure using Selectfluor® an electrophilic fluorinating agent, on cyclohexanone **2.29** in methanol, in a 78% yield.⁸¹ The product must not be kept for too long, as it is fairly unstable under nitrogen at -20°C (degrades after 10 days).

The Knoevenagel type reaction was attempted on the compound using the same method as previously,⁷³ no product **2.31** was detected and the starting material degraded. A Wittig reaction was also attempted on **2.30** to obtain **2.31** but failed as previously. The method was not applicable in order to synthesise the 2-fluorocyclohexanecarboxylic acid **2.33**. Different literature syntheses already exist to access the final compound **2.33**. This compound was not considered for feeding as its equivalent hydroxyl **1.96** had not been incorporated. Therefore the synthesis was not pursued.

2 SYNTHESIS TOWARDS FLUOROHYDRIN **CYCLOHEXANOIC ESTERS.**

Fluorohydrins are structurally closer than fluoro cyclohexane acids to the original shikimic acid pathway starter unit containing 2 hydroxyl groups in position 3 and 4 (**Figure 2.4**).

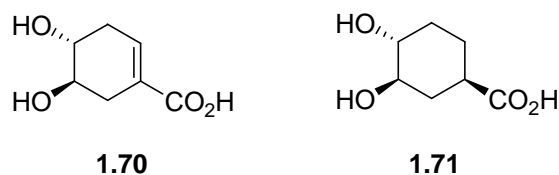


Figure 2.4: Natural starter units

Compound **2.35** has the same relative stereochemistry to compound **1.71**. It should have the best chance of incorporation compared to compound **2.34** to produce fluorinated rapamycin (**Figure 2.5**).

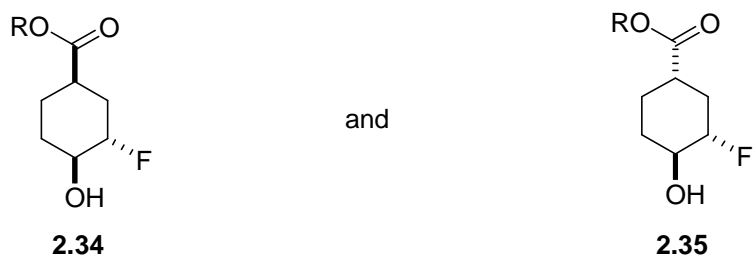
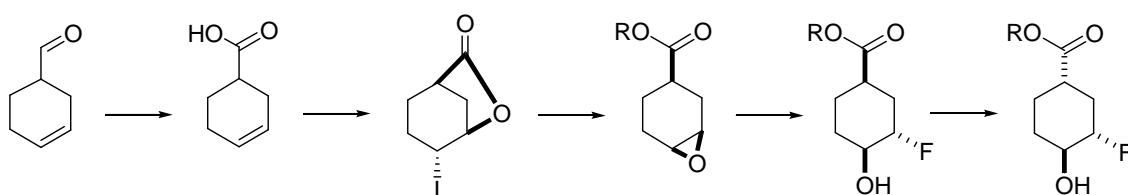


Figure 2.5: New targeted starter units

In order to access those compounds, the following synthesis was planned:

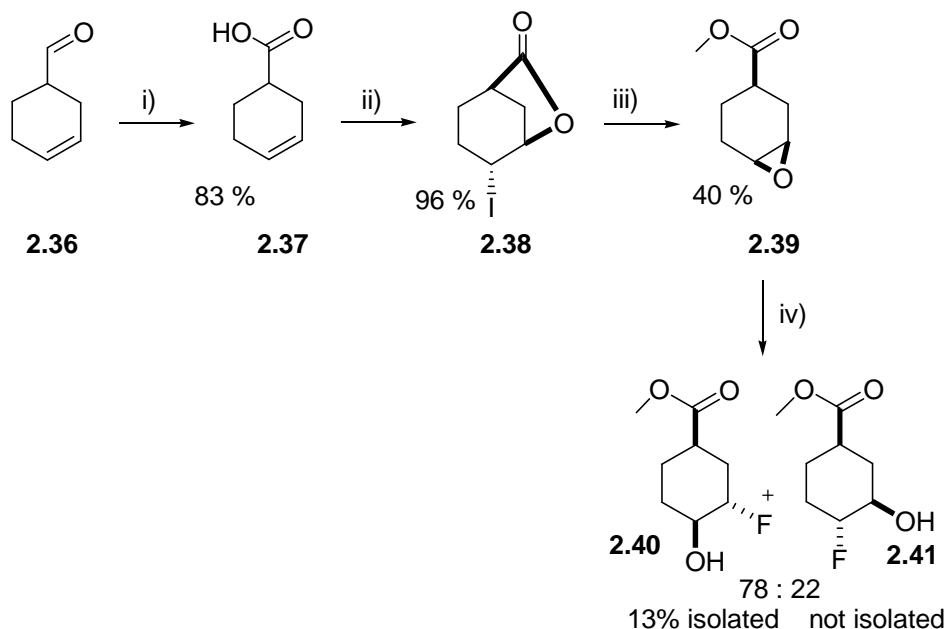


Scheme 2.20: Planned synthesis in order to obtain compound **2.34** and **2.35**.

Previous feedings of non fluorinated starter units by *Biotica Technology Ltd* suggested that incorporations were similar between the methyl and ethyl esters, and the acid of the same molecule (unpublished results). The fluorinated starter units will be fed as esters avoiding a hydrolysis step.

2.1 Synthesis towards 4-Hydroxy, 3-Fluorocyclohexanoic Methyl Ester.

2.1.1 Synthesis towards (1*R**, 3*S**, 4*S**)-methyl 3-fluoro-4-hydroxycyclohexanecarboxylate.

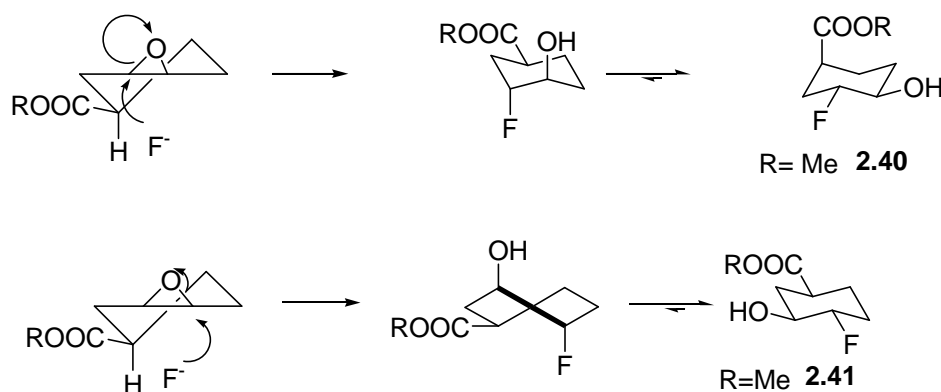


i) $\text{Ag}_2\text{O} / \text{H}_2\text{O}$, $\text{NaOH} / \text{H}_2\text{O}$, 1h, RT ii) $\text{NaHCO}_3 / \text{H}_2\text{O}$, I_2 , $\text{KI} / \text{H}_2\text{O}$ 16h, RT iii) THF, $\text{MeONa} / \text{MeOH}$, 16 h, RT iv) $(\text{HF})_3 \text{NEt}_3$ 24h, RT

Scheme 2.21: Synthetic route toward (1*R**, 3*S**, 4*S**)-methyl 3-fluoro-4-hydroxycyclohexanecarboxylate.

Cyclohex-3-enecarbaldehyde **2.36** was oxidised with silver oxide to cyclohex-3-enecarboxylic acid⁸² **2.37** (literature procedure) in a 80% yield, then reacted with potassium iodide and iodine to give (1*R**, 4*R**, 5*R**)-4-iodo-6-oxa-bicyclo[3.2.1]octan-7-one **2.38** (literature procedure) in a 96% yield.⁸³ The product was next reacted with sodium methoxide in methanol to form the epoxide (1*R**, 3*R**, 6*S**)-methyl 7-oxa-bicyclo[4.1.0]heptane-3-carboxylate **2.39** in a 40% yield.⁸³ The epoxide was opened using HF triethylamine⁸⁴ creating a mixture of fluorohydrins (1*R**, 3*S**, 4*S**)-methyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.40** and (1*R**, 3*R**, 4*R**)-methyl 4-fluoro-3-hydroxycyclohexanecarboxylate **2.41** in a 76% yield. After integration of the hydrogen adjacent to the fluorine in the ^1H NMR spectrum a ratio of 78 : 22 of **2.40** : **2.41** was found. Both compounds had very similar R_f on TLC, a slight separation was apparent after multiple developments (4 times) in DCM and ethyl acetate (95 : 5). The mixture was purified by flash chromatography on silica eluting with DCM (100%) to start and

an increasing gradient of ethyl acetate(1%, 2%, 3%, 4%, 5%max). Three columns were necessary to isolate **2.40** in a 13% yield, most of the compound remaining trapped as a mixture. The ^1H , ^{13}C , COSY and accurate mass confirmed that the isolated product was 3-fluoro-4-hydroxy-cyclohexanecarboxylic acid methyl ester **2.40**. The coupling constants of the hydrogen adjacent to the fluorine dddd, $J = 49.1, 8.3, 6.9, 3.9$ suggest that the fluorine occupies the equatorial position, deducing from the expected relative stereochemistry that the hydroxyl group also occupies the equatorial position and that the ester group occupies the axial position. This was confirmed later with the synthesis, isolation and characterisation of the ethyl ester equivalent.



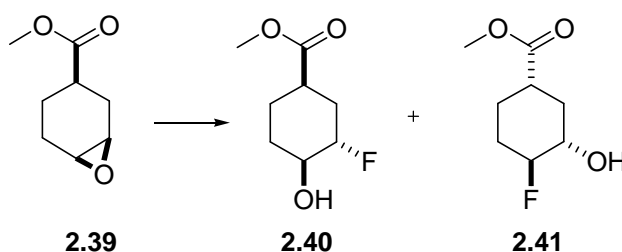
Scheme 2.22: Possible opening of the epoxide

Compound **2.40**, the major product, originates from the expected diaxial opening of the epoxide by the fluoride (see **Scheme 2.22**). The coupling constants of the hydrogen adjacent to the fluorine, in the ^1H NMR spectrum (**Table 2.13**) suggest that the fluorine group occupies the equatorial position, indicating that a chair flip occurred after formation of the fluorohydrin. This could be explained by hydrogen bonding between the hydrogen of the hydroxyl group and the fluorine, stabilising those groups in equatorial positions and lowering the global energy of the molecule. Similar bonding has been observed in cyclohexane fluorohydrins in the past.⁸⁴

In the ^1H NMR spectrum of the mixture, the coupling constants of the hydrogen adjacent to the fluorine belonging to the minor compound **2.41** (dddd, $J = 50.85, 10.38, 8.46, 5.12$ Hz) suggest that the fluorine also lies in an equatorial position. Compound **2.41** was assumed to be the all equatorial (1*R**, 3*R**, 4*R**)-methyl 4-fluoro-3-hydroxycyclohexanecarboxylate resulting from alternative opening of the epoxide. This would imply a twist boat intermediate conformation of high energy, flipping to the thermodynamically more stable all equatorial minor compound (**Scheme 2.22**). The structure of the minor compound **2.41** would later be confirmed in later experiments synthesising and isolating the ethyl ester equivalent of **2.41** **2.47**.

As both compounds were very difficult to separate alternative methods for the opening of the epoxides were investigated.

In order to selectively get **2.40**, and therefore avoiding a separation from **2.41**, different fluorinating agents (HF pyridine and HF triethylamine) were tested at different temperatures (Table 2.8).⁶⁸



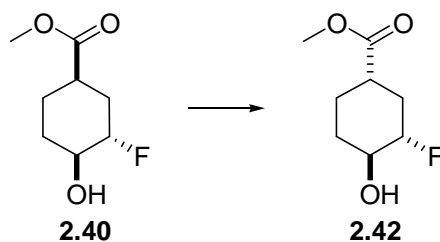
Scheme 2.23: Opening of the epoxide using fluorine as a nucleophile

Fluorinating agent	Temperature	Time of completion	Yield as pure mixture of 2.40 and 2.41	Ratio of isomers 2.40/2.41 by ¹ H NMR
HF pyridine	-78°C	2.5 h	73%	75 : 25
HF pyridine	0°C	0.5 h	36%	50 : 50
(HF) ₃ NEt ₃	20°C	20 h	76%	78 : 22
(HF) ₃ NEt ₃	5°C	72 h	73%	82 : 18
(HF) ₃ NEt ₃	-10°C to 0°C	None	No reaction	None

Table 2.8: Summary of the different attempts to open the epoxide selectively

Using HF pyridine at -78°C and at 0°C resulted in fluorination. The ratio of kinetic product **2.40** was more favourable at -78°C compared to 0°C. This result was not an improvement on the HF triethylamine fluorinating ratio. Using HF triethylamine at 5°C a slightly improved ratio of 82 : 18 between **2.40** : **2.41** was obtained. As a consequence of the lower temperature the reaction time tripled. No reaction occurred at 0°C and under. No major improvement was found towards the synthesis of compound **2.40** varying the reaction temperature and the fluorinating agent. Compound **2.41**'s ratio was best using HF pyridine as a fluorinating agent. Compound **2.41** is still present and difficult to separate, but if isolated will be fed as a new precursor. Using HF pyridine as a fluorinating agent would divert the synthesis toward production of **2.41** if necessary for a large scale feeding experiments.

2.1.2 Epimerisation attempt on the methyl ester.



Scheme 2.24: Epimerisation of the methyl ester

The epimerisation of the methyl ester compound using sodium methoxide will maintain the methyl ester. More than 1 equivalent of sodium methoxide was used because the hydroxyl proton had a lower pKa than the targeted proton adjacent to the ester group. The reaction was quenched with saturated ammonium chloride solution, then pH 7 phosphate buffer. No epimerisation was observed by TLC and NMR for either temperature (**Table 2.9**).

Reaction number	NaOMe equivalents	Temperature	Quenching temperature	Time	Result
1	4	0 °C	0 °C	6 h	No reaction
2	11	0 °C to RT	RT	4 h	No reaction

Table 2.9: Epimerisation attempt using sodium ethoxide.

At that point the only proof of relative stereochemistry was deduced from the mechanism and the coupling constants on the fluorine, no NOESY interactions were observed between the methyl group of the ester and the hydrogen adjacent to the fluorine.

Better NOESY interaction might be observed between the hydrogens on the methyl group of the ethyl ester and the hydrogen adjacent to the fluorine (**Figure 2.6**). The epimerisation could be facilitated with the ethyl group that is slightly more hindered than the methyl group and could more easily flip and stabilise itself in the equatorial position during the epimerisation. So it was decided to target the ethyl ester fluorohydrins instead and stop the investigations on the epimerisation of the methyl ester.

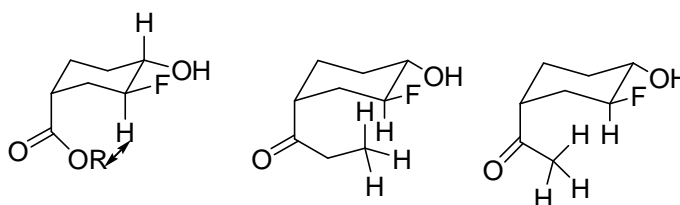
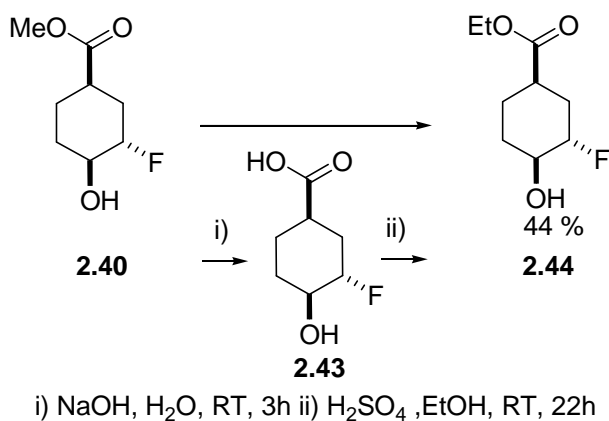


Figure 2.6: Possible NOESY interaction between CHF and H of ester group

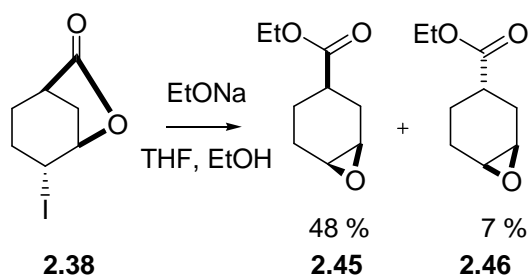
2.2 Fluorohydrin Ethyl Ester Synthesis.

2.2.1 Synthesis towards (1*R**, 3*S**, 4*S**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate.



Scheme 2.25: Conversion of the methyl ester in the methyl ester

The remaining methyl ester **2.40** was converted to the ethyl ester **2.44**. It was hydrolysed to the acid **2.43** in a sodium hydroxide aqueous solution, and esterified to the ethyl ester **2.44** using ethanol and sulphuric acid. This was performed with an overall yield of 44% over both steps (**Scheme 2.25**). This method could not be used reliably to produce gramme quantities of compound because of the difficulty to separate the mixture of both compounds **2.40** and **2.41**, and the addition of two extra steps. Therefore it was decided to redirect the synthesis towards the ethyl ester **2.44**, by opening the iodolactone **2.38** using sodium ethoxide instead of methoxide in order to access the ethyl ester epoxide **2.45**, followed by the opening of the epoxide in a similar way to that which we had previously utilised with HF triethylamine.⁸⁴



Scheme 2.26: Opening of the iodolactone

Two ethyl ester epoxides **2.45** and **2.46** were isolated from the opening of the iodolactone: the expected major compound ($1R^*$, $3R^*$, $6S^*$)-ethyl-7-oxa-bicyclo[4.1.0]heptane-3-carboxylate **2.45** (in a 48% yield) and the minor one ($1R^*$, $3S^*$, $6S^*$)-ethyl-7-oxa-bicyclo[4.1.0]heptane-3-carboxylate **2.46** (in a 7% yield) (**Scheme 2.26**). On the TLC using an 80 : 20 petroleum ether : ethyl acetate system the minor epoxide **2.46** had an R_f of 0.42 and the major one **2.45** of 0.34. The stereochemistry of the major epoxide **2.45** and the minor epoxide **2.46** was confirmed by NOESY NMR. For the minor epoxide a lack of interaction between the proton adjacent to the ester and the proton adjacent to the epoxide was observed (see **Figure 2.7**). This was in contrast to the interactions observed for the major epoxide.

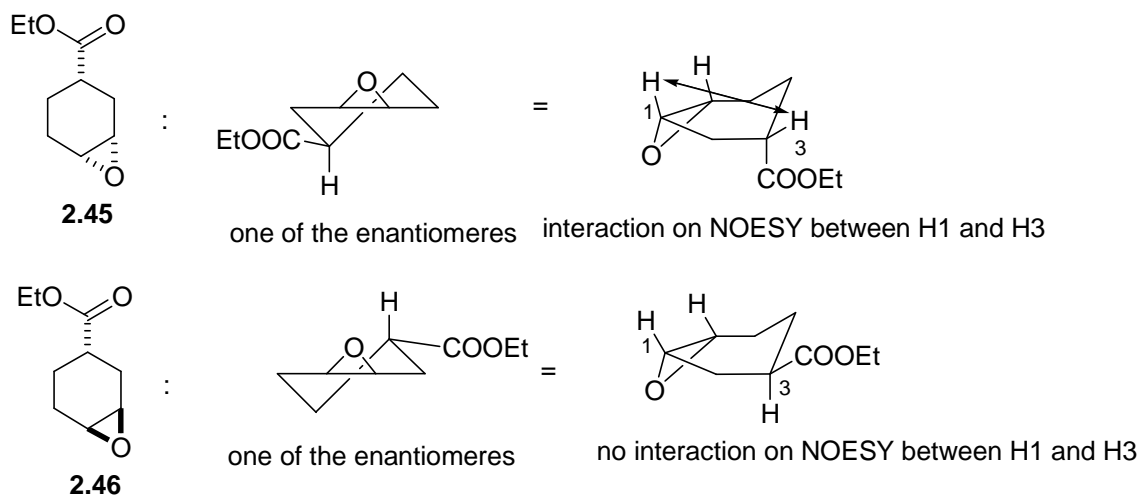
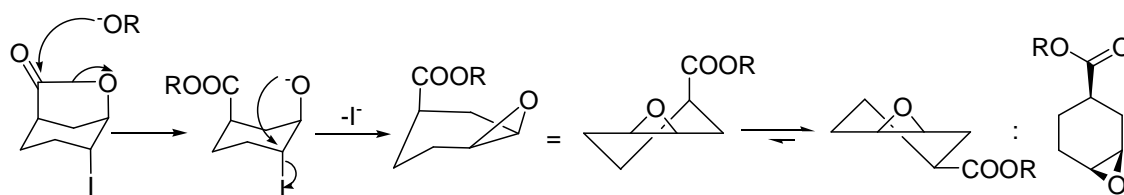


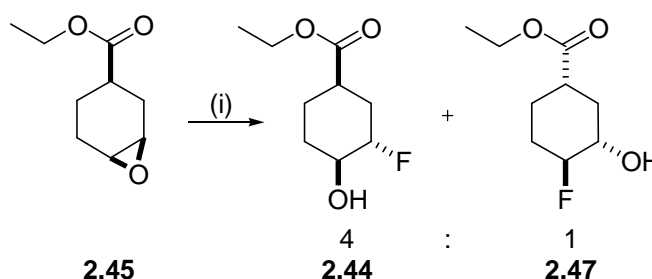
Figure 2.7: Stereochemistry of both epoxides minor and major showing characteristic observed and non observed NOESY interactions.

The formation of the major epoxide can easily be explained (**Scheme 2.27**), but the formation of the minor epoxide is surprising. As sodium ethoxide is present in the reaction one plausible explanation for the formation of the minor epoxide is epimerisation on the carbon adjacent to the ester of part of the main epoxide. In order to confirm this epimerisation, the major epoxide was placed in ethanol with sodium

ethoxide, and after a few hours minor epoxide formed, confirming the origin of the minor epoxide.



Scheme 2.27: Stereochemistry of the major epoxide formation step of one of the enantiomers.



(i) $\text{Et}_3\text{N HF}$, RT, 16 h, 76 % as a mixture of **2.44** and **2.47**

Scheme 2.28: Opening of the ethyl ester

Epoxide ($1R^*$, $3R^*$, $6S^*$)-ethyl 7-oxa-bicyclo[4.1.0]heptane-3-carboxylate **2.45** was opened with HF triethylamine⁸⁴ to yield a mixture of both isomers ($1R^*$, $3S^*$, $4S^*$)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** and ($1S^*$, $3S^*$, $4S^*$)-ethyl 4-fluoro-3-hydroxycyclohexanecarboxylate **2.47** in a 4 : 1 mixture. This mix of both compounds was isolated after a first fast flash chromatography on silica column in a 76% yield. At first TLC revealed both compounds only as one same spot. After using multiple developments TLC (after 3 developments) 2 spots running very close could be identified. The separation using flash chromatography only resulted in very partial separation, most of the compound staying as a mixture. The major fluorohydrin **2.44** was only isolated in a 10% yield after multiple columns, the rest of the compound staying as a mixture of **2.44** and **2.47** like the previous methyl ester compounds **2.40** and **2.41**. In order to obtain a crystal structure to confirm the relative stereochemistry of compound **2.44**, a *p*-nitro benzoyl ester derivative **2.48** of the alcohol was synthesised. The PNB derivative **2.48** was obtained in a 63% yield, by reacting the original fluorohydrin **2.44** with 4-nitro-benzoyl chloride in pyridine.⁸⁵ The crystal structure of **2.48** demonstrates relative orientations of the fluorine, hydroxyl group and ester group on the ring and confirms the relative stereochemistry of **2.44** (**Figure 2.8**).

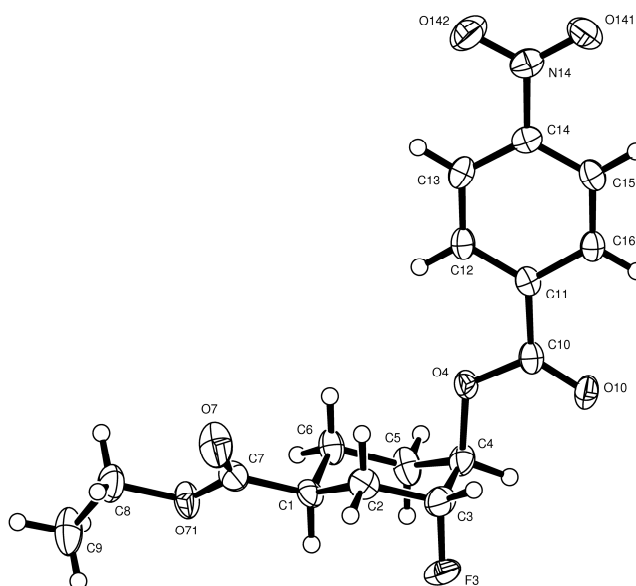
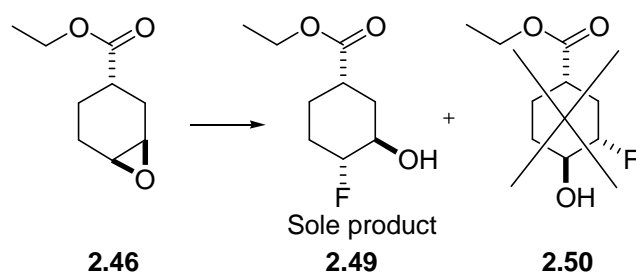


Figure 2.8: X-ray structure of (1*S**, 2*S**, 4*R**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl 4-nitrobenzoate **2.48**

In the ^1H NMR spectrum of the original fluorohydrin **2.44**, the coupling constants of the hydrogen adjacent to the fluorine and the NOESY interactions suggested that the fluorine and the hydroxyl group are in equatorial positions, and the ethyl ester in the axial position (see **Table 2.13**). This conformation is possibly stabilised by hydrogen bonding between the hydrogen of the hydroxyl group and the fluorine as previously mentioned.

The NOESY interaction between the hydrogen of the CH_3 in the ethyl group and the hydrogen adjacent to the fluorine, confirmed that the ethyl ester compound **2.44** was easier to work with than the methyl ester equivalent **2.40** that had no NOESY interaction to confirm the stereochemistry.



Scheme 2.29: Opening of the alternative epoxide

The alternative epoxide was opened in a similar way using HF triethylamine.⁸⁴ The opening of the epoxide resulted in the formation and isolation of the expected

fluorohydrin (1*R**, 3*S**, 4*S**)-ethyl 4-fluoro-3-hydroxycyclohexanecarboxylate **2.49** resulting from the diaxial opening of the epoxide. Sufficient quantities of fluorohydrin **2.49** were synthesised to enable feeding studies with *S. hygroscopicus* MG-210 in order to produce another fluorinated prerapamycin analogue.

In order to obtain a crystal structure to confirm the relative stereochemistry of the compound a *p*-nitro benzoyl ester derivative of the alcohol was synthesised. The PNB derivative **2.51** was obtained in a 59% yield, reacting the original fluorohydrin with 4-nitro-benzoyl chloride in pyridine.⁸⁵ The relative stereochemistry of the compound was confirmed (**Figure 2.9**).

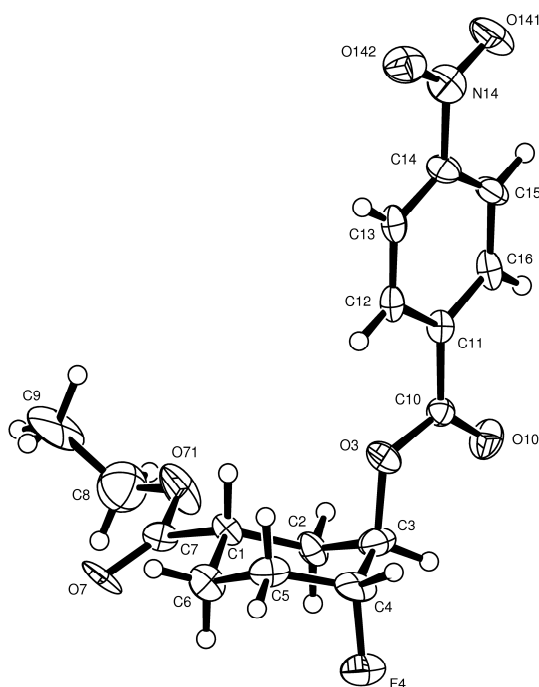


Figure 2.9: X-ray structure of (1*S**, 2*S**, 5*R**)-5-(ethoxycarbonyl)-2-fluorocyclohexyl 4-nitrobenzoate **2.51**

In the ¹H NMR spectrum of the original fluorohydrin the coupling constants of the hydrogen adjacent to the fluorine and the NOESY interactions, suggested that the fluorine occupies the equatorial position, the hydroxyl group occupies also the equatorial position, and the ethyl ester the axial position (see **Table 2.13**). This conformation is probably stabilised by hydrogen bonding between the hydrogen of the hydroxyl group and the fluorine as previously discussed.

Alternative opening

In order to form the unfavourable all equatorial fluorohydrin **2.50**, the product has to go through the very high energy double twisted boat transition state. Therefore an

alternative fluorinating agent was tested: HF pyridine at various temperatures from -78 °C to room temperature but only compound **2.49** was obtained.

2.2.2 THP protection and optimisation of the separation between both isomers.

In order to improve the separation between both isomers **2.44** and **2.47**, it was decided to explore the introduction of a THP protecting group on the alcohol. By adding a THP group, an extra stereocenter is created, forming two diastereoisomers for each original molecule (**Figure 2.10**).

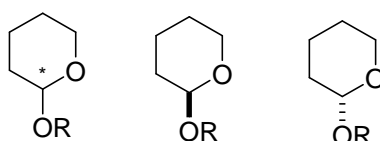
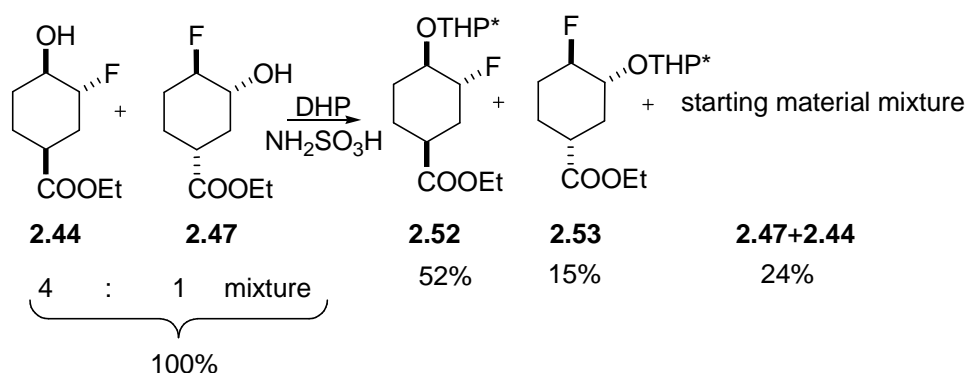
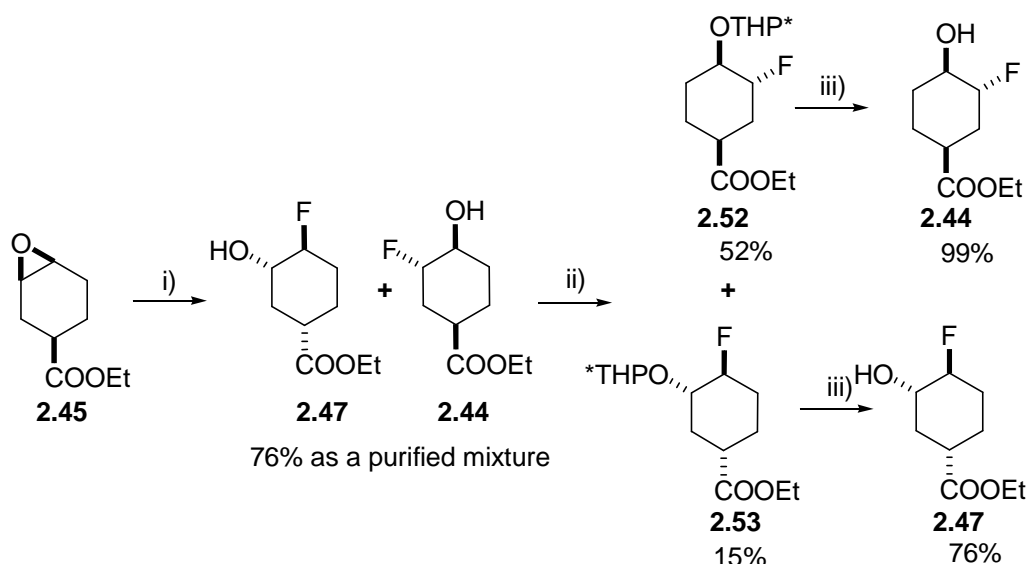


Figure 2.10: THP protecting group



Scheme 2.30: THP protection.⁸⁶

The purified mixture of **2.44** and **2.47** was stirred in 3, 4-dihydro-2H-pyran (DHP) with a sulfamic acid catalyst for 4 days.⁸⁶ The reaction did not go to completion, 24% of the starting material was recovered (DCM : Ethyl acetate 95 : 5 solvent system R_f 0.30). The mixture of diastereoisomers THP derived from the protection of the major compound **2.52** was isolated after 3 long separations using column chromatography in a 52% yield and the diastereoisomeric mixture of the THP protected minor compound **2.53** was isolated in a 15% yield (**Scheme 2.30**). Both diastereoisomers of the main derived isomer were isolated in very small quantities and were characterised separately (DCM : Ethyl acetate 95 : 5 solvent system R_f 0.51 and 0.48), the main part of the mixture was inseparable. The minor mixture was only characterised as a mixture (DCM : Ethyl acetate 95 : 5 solvent system R_f 0.37).



i) NEt_3 , 3HF, RT, 40 h ii) DHP, $\text{NH}_2\text{SO}_3\text{H}$, RT, 4 days iii) *p*-TsOH, EtOH, RT, 2h

Scheme 2.31: Protection, separation and deprotection using a THP group

The major mixture was hydrolysed with *p*-TsOH in ethanol in a 99% yield, and the minor mixture in a 76% yield.⁸⁷

The overall yield from the epoxide **2.45** to the isolated compound **2.44** over the fluorination, THP protection, and deprotection is equivalent to 39%. This compares favourably to the previous 10% yield obtained (fluorination followed by a very difficult and incomplete separation). The THP protection of the alcohol allowed a better separation quadrupling the amount of isolated (1*S**, 3*R**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** and isolating for the first time the all equatorial (1*S**, 3*S**, 4*S**)-ethyl 4-fluoro-3-hydroxycyclohexanecarboxylate **2.47** that can directly be fed to *S. hygroscopicus* as an all equatorial shikimic acid fluorinated analogue (**Figure 2.11**).



Figure 2.11: Shikimic acid derivative **1.71** and fluorinated analogue **2.47**.

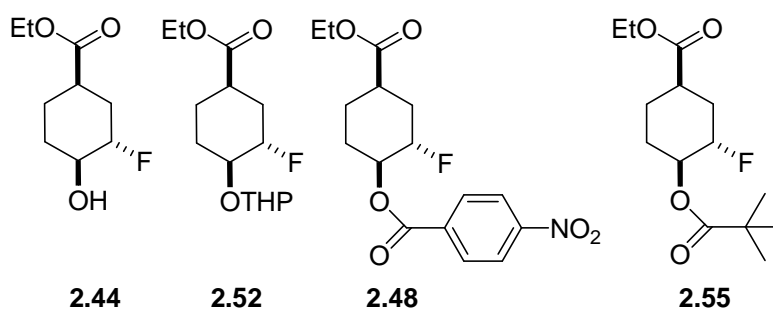
Following the successful isolation of the minor fluorohydrin **2.47**, a *p*-nitro benzoyl ester derivative of the alcohol was synthesised in order to obtain a crystal structure to confirm the relative stereochemistry. The fluorohydrin **2.47** was reacted with 4-nitrobenzoyl chloride in pyridine.⁸⁵ The PNB derivative **2.54** was obtained in a 63% yield. The compound would not crystallize and therefore no X-ray structure was available. In

the ^1H NMR spectrum of the original fluorohydrin **2.47** the coupling constants of the hydrogen adjacent to the fluorine, the hydroxyl group and ethyl ester and the NOESY interactions suggest that the fluorine, the hydroxyl group and the ethyl ester are all in equatorial positions (see **Table 2.13**). This conformation is the most stable, thermodynamically.

2.2 Epimerisation of the Ethyl Ester

As the first attempt of epimerisation on the methyl ester was unsuccessful, it was decided to try the epimerisation on the ethyl ester as well as a range of derivatives of the alcohol. The THP alcohol derivative **2.52** and the PNB derivative **2.48** had already been synthesised. A pivalic ester derivative of the alcohol **2.55** was also synthesised. It was believed that the bulky substituent would preferentially occupy the equatorial position thus forcing the ethyl ester to occupy the axial position. This could add selectivity towards the formation of the epimerised compound

The pivalic ester **2.55** was prepared in one pot by combining pivalic acid and thionyl chloride in DCM yielding pivaloyl chloride. This was reacted with compound **2.44** to form (1*R**, 3*S**, 4*S**)-ethyl 3-fluoro-4-(pivaloyloxy)cyclohexanecarboxylate **2.55** in a 35% It was believed that such a bulky substituent would position itself in equatorial.



Scheme 2.32: Range of compounds tested for epimerisation.

The epimerisation was first attempted, using one equivalent of sodium ethoxide for the THP, PNB and pivalic acid derivatives and two equivalents for the free alcohol compound, because of the deprotonation of the alcohol in the alkoxide. The reactions were attempted at -20°C and room temperature. The reactions were quenched using pH 7 phosphate buffer. No epimerised compound was detected.

2.2.1 Epimerisation kinetic conditions

Previously sodium methoxide was used unsuccessfully in order to epimerise the methyl fluorohydrins. Sodium hydride a stronger base was used next for deprotonation purposes in THF at -20°C. The reaction was quenched with sterically hindered proton donors also introduced at -20°C. Phthalamide and *tert*-butanol were used. No epimerisation was observed using *tert*-butanol introduced at -20 °C in diethyl ether possibly because of solubility problems, but using phthalamide some epimerisation occurred on the non protected molecule (**Table 2.10**). After a scale up of the reaction and separation using two successive columns, 63% of the starting material was recovered and the epimerised compound was isolated in a 23% yield.

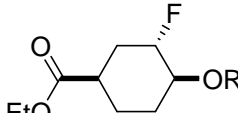
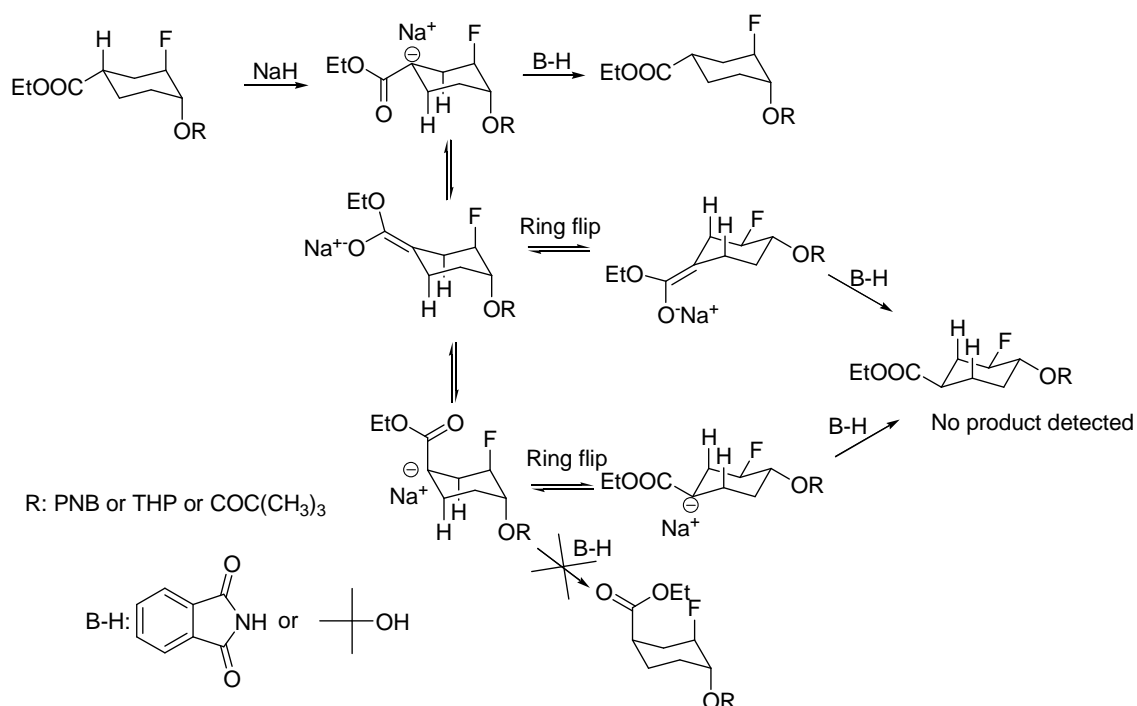
Compound 	¹ H Coupling constants of H adjacent to the fluorine	Suggested stereochemistry of the OR, fluorine and ester group in the starting material	Epimerisation Ratio (%)
R: H	49.1, 8.1, 6.8, 3.9	e, e, a	23
R: THP mixture of 2 Diastereoisomers	47.9, 6.0, 6.0, 3.4 47.2, 4.8, 4.8, 2.7	a, a, e a, a, e	0
R: PNB	47.4, 6.4, 6.1, 3.5	a, a, e(confirmed by X-ray structure)	0
R: COC(CH ₃) ₃	47.0, 4.6, 4.3, 4.3	a, a, e	0

Table 2.10: Correlation between conformation of the starting material and epimerisation in kinetic conditions.

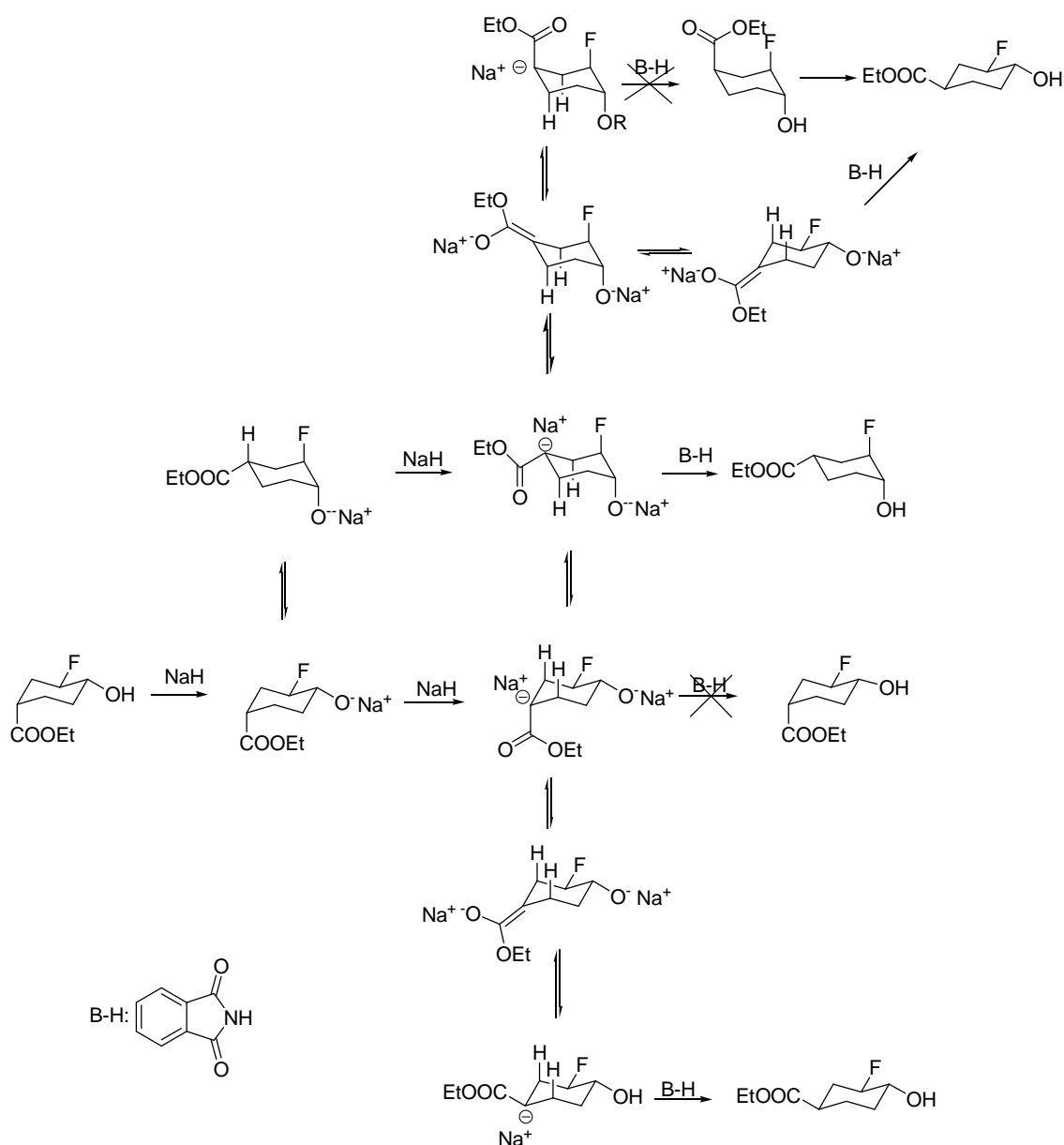
Surprisingly the epimerisation was only successful on the non protected alcohol. In the ¹H NMR spectrum of the THP derivative the coupling constants of the proton adjacent to the fluorine, and X-ray structure of the PNB derivative suggest that both compounds have their fluorine group in the axial position, resulting in axial derivatised alcohols and equatorial ethyl esters. The proton NMR spectrum of the pivalic derivative was then investigated. It was expected that the sterically hindered pivalic ester would occupy the equatorial position, leaving the ethyl ester in the axial position. However the coupling constants of the proton adjacent to the fluorine indicate that the fluorine occupies the

axial position, therefore indicating that the pivalic ester occupies the axial position and the ethyl ester the equatorial position as with both previous derivatised compounds.



Scheme 2.33: Possible mechanisms explaining the epimerisation or non epimerisation in kinetic conditions with the ethyl ester occupying the equatorial position in the starting material.

The sterically hindered OR group and fluorine group in an axial position are not favourable to the epimerisation (**Scheme 2.33**). Both axial hydrogens of the cyclohexane ring adjacent to the desired epimeric centre, and the axial sterically hindered OR group in the case of the PNB, THP and pivalic ester derivative sterically hinder the approach of the bulky proton donor in equatorial (see **Scheme 2.33** and **2.34**). The favourable axial approach leads to the non epimerised starting material (**Scheme 2.33**). A ring flip, putting the fluorine and the hindered OR group into the equatorial position, would be the only way the epimerised product would be favoured. It is unlikely but the low temperature kinetic conditions might be unfavourable to this ring flip, and could explain the lack of epimerised compound. To investigate this possibility the reactions were repeated at a higher temperature of 0 °C, but the starting material degraded (cleavage of the fluorine). The most probable explanation would be that the deprotonation on the epimeric carbon did not occur at -20°C.



Scheme 2.34: Mechanism of the epimerisation of **2.44** under kinetic conditions.

In the case of the free alcohol, the ethyl ester occupies the axial position. This conformation is favourable due to the approach of the sterically hindered proton donor phthalamide, to give the epimerised compound (**Scheme 2.34**). The approach on the opposite side resulting in the starting material is unfavourable. Both protons in the axial position adjacent to the desired epimeric centre hinder the approach of the bulky proton donor (**Scheme 2.34**). A majority of epimerised compound was expected, but the epimerisation only appeared in a 23% yield. 70% of starting material was recovered with traces of phthalamide. However separating the phthalamide was very difficult and the yields were not reproducible. The fact that over 70% of starting material did not epimerise could be rationalised from the first deprotonation of the alcohol group. This would have stopped the stabilising hydrogen bonding between the alcohol group and the

fluorine. Without this bonding, the ethyl ester would want to position itself in equatorial (**Scheme 2.34**). Even at -20°C it is possible that part of the product ring flipped before the deprotonation of the carbon in alpha of the ester. Another explanation would be that only part of the starting material was deprotonated on the epimeric centre.

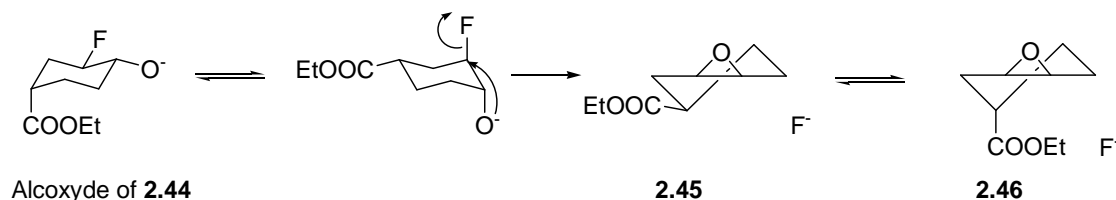
As the epimerisation using sodium hydride in kinetic conditions was unsuccessful another base, *n*-BuLi in THF, was used in similar kinetic conditions from -78°C to -20°C. No epimerised compound was detected but some degradation was observed. As the kinetic conditions were not satisfactory (the yield being fairly low and not always repeatable), it was decided to test different bases and an acid in thermodynamic conditions: *t*-BuOK, EtONa, pyridine and *p*-TsOH. The all equatorial epimerised compound is the more thermodynamically stable. All experiments were followed by TLC to track the formation of the epimerised compound. The temperature was increased every 20 minutes by 10°C (**Table 2.11**).

Reagent	Number of equivalents	Solvent	Temperature range of reactions (°C)	Identified Side products	Degradation	Presence of epimerised compound
<i>p</i> -TsOH	Catalytical	Toluene	20-110	No	Yes	0
Pyridine	1.3	Ethanol	20-79	No	Yes	0
	1.3	Toluene	20-110	No	Yes	0
EtONa	1.2-1.4	Ethanol	20-79	Epoxides acids	Yes	Traces on TLC
<i>t</i> -BuOK	1.3	Toluene	20-110	Epoxides acids	Yes	Yes
None	0	Toluene	110	No	No	0

Table 2.11: Range of epimerisation attempts of **2.44** using various reagents with thermodynamic conditions.

Epimerised compound **2.50** was observed with ethoxide in ethanol by TLC in very low concentration, but after work up, a mixture of mainly both epoxides **2.45** and **2.46** obtained previously (opening of the iodolactone), and starting material was isolated. The epimerised compound seems to be relatively volatile and during the work up traces of epimerised compound might have evaporated with the ethanol as an azeotrope.

Epimerisation of **2.44** only started occurring in thermodynamic conditions using potassium *tert*-butoxide between 70 and 110 °C (reflux). The highest epimerisation rate was observed at reflux. However some degradation and side products formed even at lower temperatures. Both epoxides **2.45** and **2.46** that had formed as expected products in the opening of the iodolactone previously (**Scheme 2.35**) were also identified as side products.



Scheme 2.35: Side reaction formation of the epoxides.

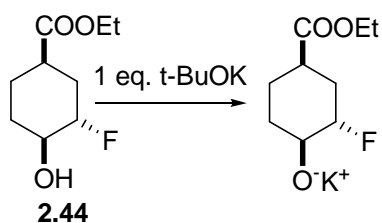
The formation of the epoxide can only proceed if the alkoxide and the fluorine are in the axial position. This suggests that part of the molecules as an alkoxide ringed flip to the diaxial fluorine alkoxide, due to the breaking of the hydrogen bond.

The reaction was left to cool down then exposed to the moisture in the air, when quenched a small dark solid formed. This solid was not soluble in CDCl_3 , but was solubilised in CD_3OD and analysed by NMR. The ^1H NMR spectrum identified a mixture of acids resulting in the hydrolysis of the ethyl ester of both epoxides and starting material. This hydrolysis could be due to hydroxide formed from the 1.2 equivalents of *tert*-butoxide coming into contact with moisture in the air.

2.2.2 Thermodynamic epimerisation using potassium *tert*-butoxide.

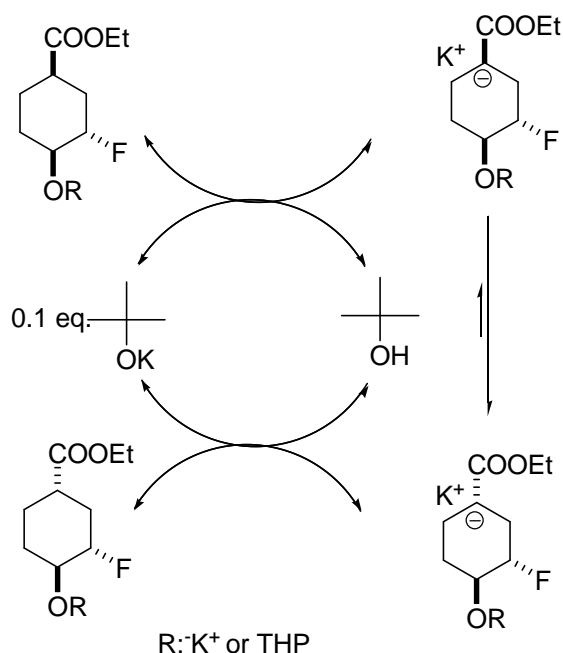
As the pKa values of *tert*-butanol (29.4 in DMSO) and the hydrogen on the desired epimeric centre are very close (approximately 29.5 in DMSO), the deprotonation will become reversible if the reaction is carried out at a high temperature (toluene reflux, thermodynamic conditions) and the equilibrium between non epimerised compound **2.44** and epimerised compound **2.50** should displace itself strongly toward the thermodynamically more stable compound, accumulating the epimerised compound **2.50**.

In order to epimerise the fluorohydrin **2.44**, one equivalent of base is necessary to deprotonate the hydroxyl group (lower pKa than the protons on the desired epimeric centre and *tert*-butanol) and a catalytic amount of base is necessary for the epimerisation (0.1-0.2 equivalents).



Scheme 2.36: Deprotonation of the hydroxyl to the alkoxide.

The thermodynamic conditions can be compared to a catalytic cycle using 0.1 to 0.2 equivalents of base (**Scheme 2.37**).

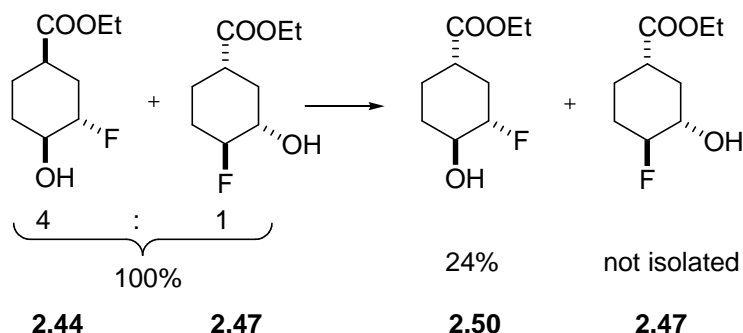


Scheme 2.37: Catalytic mechanism of the thermodynamic epimerisation.

The reaction was repeated on fluorohydrin **2.44** using different temperatures (between 70 and 110°C), different amounts of potassium (*tert*-butoxide between 1.1 and 1.5 equivalents) and different reaction times (between 10 and 60 minutes). The best yield was obtained at reflux for 30 minutes, with 1.2 equivalents of potassium *tert*-butoxide with a yield of 34%. After 30 minutes at reflux the amount of degradation products increases very quickly (after 50 minutes most of the starting material and the product would have degraded). This makes the reaction quite difficult to reproduce in a reasonable yield due to the starting material and the product degrading during the reaction.

Previously the opening of the epoxide gave a 4 to 1 mixture of two isomers **2.44** and **2.47** that were very difficult to separate and had to be protected with a THP group in

order to facilitate the separation, followed by a deprotection. The unwanted isomer is already all equatorial and should not epimerise (thermodynamically more stable than if epimerised).



Schemes 2.38: Epimerisation of the mixture.

The mixture was epimerised and after optimisation a total yield of 24% was obtained avoiding the protection and deprotection step. As the starting mixture had a ratio of 4 to 1 this means that 30% of product **2.44** epimerised.

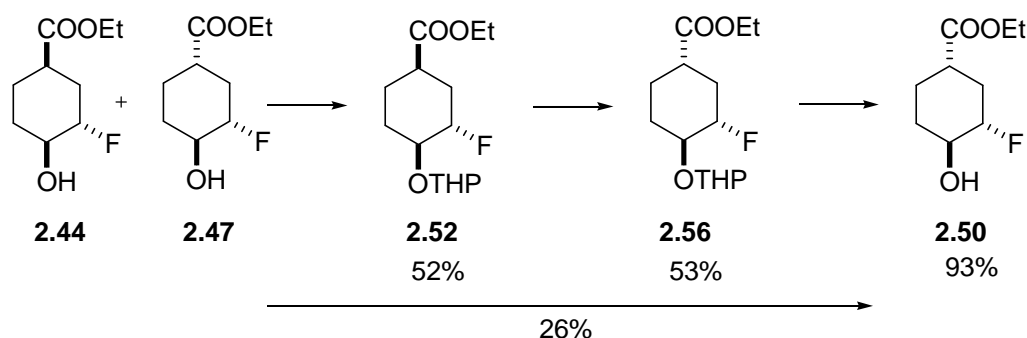
There are limits to this method, however as part of the product degrades and conditions for this reaction are not easy to reproduce. Just a few minutes extra reaction time reduces the yield considerably, and the entire product and the starting material could degrade if the time is doubled. This reaction is also difficult to follow by TLC as both compounds of the original mixture have very similar R_f , only multiple development can separate them.

The epimerisation on the free alcohol has 3 major disadvantages:

- 1- The formation of the epoxides as side products.
- 2- Using more than 1 equivalent of *t*-BuOK per molecule due to the presence of the proton on the hydroxyl group. This excess base in contact with water produces hydroxides potentially responsible for some of the hydrolysis of the esters and acids and other degradation. Boiling the compound in dry toluene without presence of base showed no degradation.
- 3- The direct production of the more volatile epimerised compound in a high boiling point solvent toluene. The evaporation of the solvent *in vacuo* has to be done very carefully at a low temperature, and controlled reduce pressure.

Kinetic conditions (low temperature) had failed previously because of the conformation of the protected molecules (the ethyl ester standing in the equatorial position). In thermodynamic conditions the ring could flip and epimerisation could occur. The thermodynamic conditions were tried on the THP protected diastereoisomeric mixture, which was originally produced for separation purposes. As a result of using 0.2

equivalents of *t*-BuOK in toluene at reflux for 40 minutes the compound epimerised in a 53% yield, and 46% of starting material was recovered. Little degradation was observed on TLC, only appearing after an hour reflux. The deprotection was performed with *p*-TsOH in methanol, instead of ethanol because of the volatility issues with the deprotected product; this resulted in a 93% yield.⁸⁷ When ethanol was used as a solvent in the deprotection and removed *in vacuo* some compound was lost, because of its volatility.



Scheme 2.39: Epimerisation of the THP mixture.

Compared to the previous epimerisation, the reaction results are easily reproducible. Nearly all the remaining starting material was recovered. The overall yield is slightly improved, 26% over 3 steps instead of 24% yield previously achieved. This method compared to the previous one is fully reproducible, the starting materials are recovered and the product does not degrade under the conditions used. The starting material can also be recycled. The THP protection is not an added step as it is necessary for the separations of both isomers (compounds have to be fed as a pure racemic mixture). This method was chosen for the gramme scale synthesis for quantitative feeding of the all equatorial compound.

A PNB derivative of **2.50** was synthesised as previously described. The relative stereochemistry of epimerised fluorohydrin **2.50** was confirmed by X-ray structure of the PNB derivative **2.57**, all 3 groups positioned in equatorial (**Figure 2.12**).

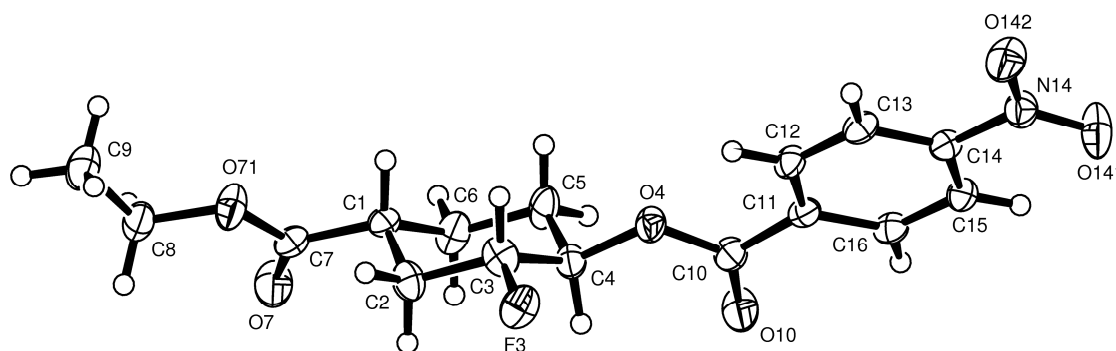


Figure 2.12: X-ray structure of (1*S**, 2*S**, 4*S**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl 4-nitrobenzoate **2.57**

At that point in time four fluorohydrins had been synthesised (**Figure 2.13**).

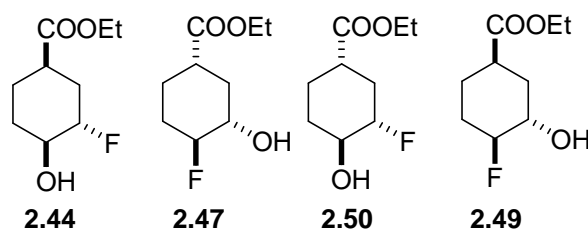


Figure 2.13: Range of fluorohydrins synthesised.

Qualitative feeding indicated that only the epimerised compound **2.50** was well incorporated. For a better understanding about the incorporation of the fluorohydrins by *S. hygroscopicus*, two more molecules were targeted **2.58** and **2.59** (**Figure 2.14**).

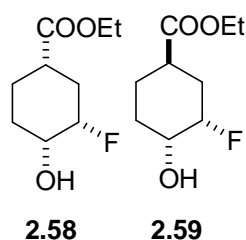
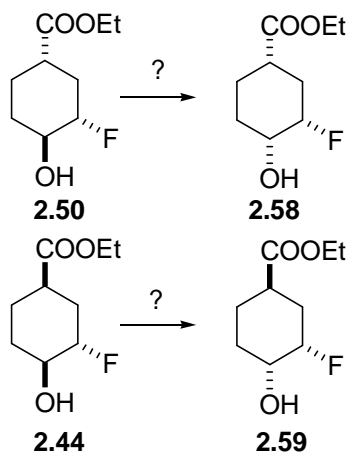


Figure 2.14: New targets.

In order to access compounds **2.58** and **2.59**, the hydroxyl group of the non epimerised **2.44** and the epimerised compound **2.50** need to be inverted.

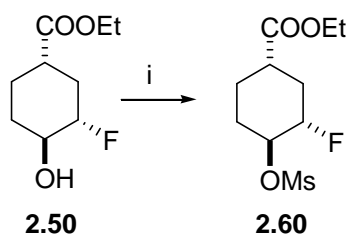
2.3 Alcohol Inversion



Scheme 2.40: Inversion of the hydroxyl group.

The Mitsunobu reaction is a classic method for inverting a hydroxyl group. However it is generally not very successful on alcohols in a sterically hindered environment. The inversion was attempted on the less sterically hindered all equatorial fluorohydrin **2.50** without any success, since no reaction occurred, and the starting material was recovered.⁸⁸

Alternatively in order to invert the hydroxyl group, the hydroxyl needs to be transformed into a good leaving group, allowing it next to be displaced through an $\text{S}_{\text{N}}2$ mechanism by a hydroxyl group or a group that can be hydrolysed to a hydroxyl group. Mesyl chloride was first used in order to transform the hydroxyl group into a good leaving group ($-\text{OMs}$).



i) MsCl , DMAP, Hunig's base, DCM, -10°C , 60%

Scheme 2.41: Mesylation step of the alcohol.

Fluorohydrin **2.50** was transformed using mesyl chloride to (1*S**, 3*S**, 4*S**)-ethyl 3-fluoro-4-(mesityloxy)cyclohexanecarboxylate **2.60** in a 60% yield.⁸⁹

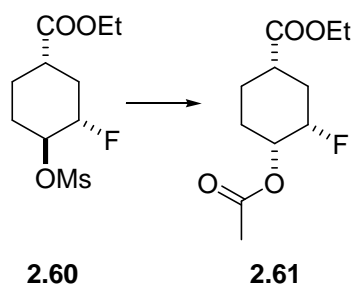
In order to displace the mesylate group, cesium acetate,⁸⁹⁻⁹¹ sodium acetate,⁹² were tried at various temperatures, and in some cases under microwave conditions (**Table 2.12**). In order to displace the hydroxyl group, formic acid⁹³ was tried on the free alcohol **2.50** (**Table 2.12**)

Reactive	Solvent mixture	T (°C)	Problems occurring	time	Product detected	Predicted group in product
CsOAc ^a	18-Crown-6 Toluene	Reflux 110	No reaction	24h	None	Acetate
CsOAc ^a	18-Crown-6 Toluene	Microwave 250	Explosion or degradation	10 min.	2.61 traces	Acetate
NaOAc ^a	DMF H ₂ O	Reflux 120	Does not go to completion	3 days	2.61 12% isolated	Acetate
NaOAc ^a	DMF	Microwave 250	Explosion		2.61 traces	Acetate
HCOOH ^b DCC CuCl DMAP	DCM	Reflux 40		3 days	None	formate or OH.

^a: inversion on mesylate **2.60**, ^b: inversion on free alcohol **2.50**.

Table 2.12: Main inversion attempts on the mesylate **2.60** and the free alcohol **2.50**.

The microwave reactions would lead some times to unpredictable explosions due to a sudden rise in pressure in the vial, whilst using acetate salts, this possibly due to decarboxylation. The best result was obtained when the mesylate **2.60** was heated at reflux with sodium acetate in an 80 :20 mixture of DMF and water.⁹² However the best yield observed for the isolated acetate compound was limited to 12% after optimisation.



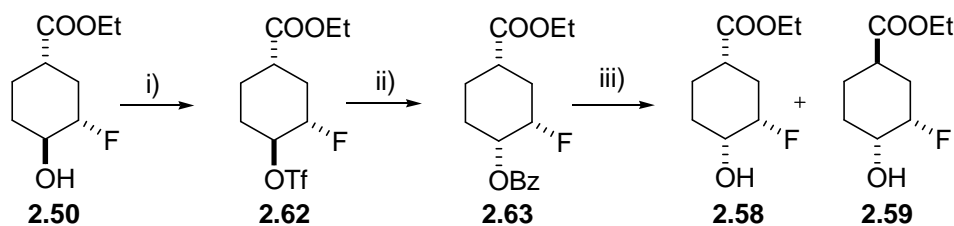
i) NaOAc, DMF : H₂O 8 : 2 , reflux, 12%

Scheme 2.42: Displacement of the mesylate with sodium acetate

The reflux temperature and the solubility of sodium acetate are a limiting factor. A better leaving group is needed than the mesylate.

In sugar chemistry the most activated leaving group used for inverting alcohol groups is the triflate. The lipophilic tetrabutylammonium benzoate would be a very good nucleophile in an aprotic solvent such as toluene. Benzoate also has the advantage to be

UV active (easily followed by TLC) and a solvent like toluene can be heated at reflux (110°C) if necessary.

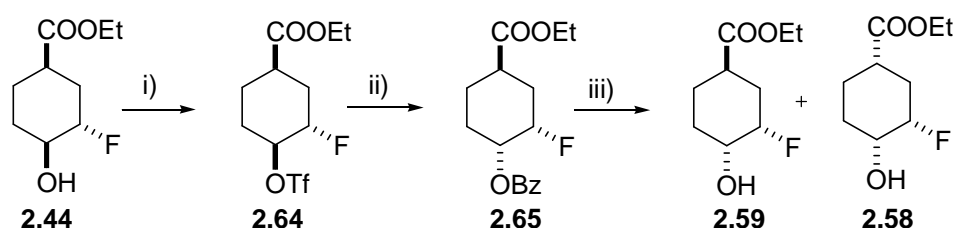


(i) Triflic Anhydride, Pyridine, DCM, 0°C, 2 h, 67% (ii) $\text{Bu}_4\text{N}^+\text{BzO}^-$, toluene, RT, 16 h, 61 %
 (iii) Na, EtOH, RT, 16 h, 88 % **2.58**, 4 % **2.59**.

Scheme 2.43: Alcohol inversion of **2.50** through a triflates

(1*S**, 3*S**, 4*S**)-Ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.50** was transformed using triflic anhydride to (1*S**, 3*S**, 4*S**)-ethyl 3-fluoro-4-(trifluoromethylsulfonyloxy)cyclohexanecarboxylate **2.62** in a 67% crude yield. The compound was not purified at that stage because of stability issues with the triflate. The triflate was then displaced and inverted using tetrabutyl ammonium benzoate to (1*R**, 2*S**, 4*S**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl benzoate **2.63** in a 61% yield. Finally the alcohol was deprotected using ethoxide to (1*S**, 3*S**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.58** in an 88% yield. Some epimerised compound (1*R**, 3*S**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.59** was isolated in a 4% yield.

The same method was applied toward the synthesis of (1*R**, 3*S**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate.



(i) Triflic Anhydride, Pyridine, DCM, 0°C, 2 h, 76 % (ii) $\text{Bu}_4\text{N}^+\text{BzO}^-$, toluene, RT, 16 h, 65 %
 (iii) Na, EtOH, RT, 16 h, 57 % **2.59**, 33 % **2.58**

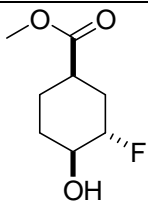
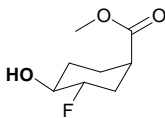
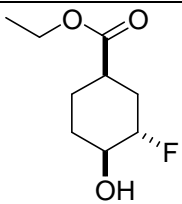
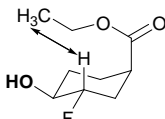
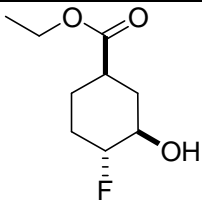
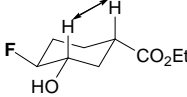
Scheme 2.44: Alcohol inversion of **2.44** through a triflate

(1*R**, 3*S**, 4*S**)-Ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** was transformed to (1*R**, 3*S**, 4*S**)-ethyl 3-fluoro-4-(trifluoromethylsulfonyloxy)cyclohexanecarboxylate **2.64** in a 76% crude yield (not purified for stability issues as previously), then was inverted using to (1*R**, 2*S**, 4*R**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl benzoate **2.65** in a 65% yield. Finally the alcohol was deprotected to (1*R**, 3*S**, 4*R**)-

ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.59** in a 57% yield, and a large amount of epimerised compound (1*R**, 3*S**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.58** was isolated in a 33% yield.

2.4 Confirmation of the Stereochemistry of Fluorohydrin cyclohexane Methyl and Ethyl Esters.

All the fluorohydrins were assigned using ^1H , ^{13}C , DEPT, COSY, HSQC and NOESY NMR. Table **2.13** shows the coupling constants found in the ^1H NMR spectrum of hydrogens situated on the stereocenters, when available, and the NOESY NMR interactions, also when available (Table **2.13**). This allows us to deduce the relative stereochemistry of the racemic mixtures in CDCl_3 . Only one of the enantiomers is represented in the final column.

Molecule	Coupling constants of H adjacent to the F OH COOR	All NOESY Interaction Between H adjacent to F, OR, COOEt and CH_3	Deduced stereochemistry with observed NOESY interactions
 2.40	CHF : dddd, $J = 49.1$, 8.3, 6.9, 3.9 Hz CHOH : m CHCOOMe : m	No interaction	
 2.44	CHF : dddd, $J = 49.1$, 8.1, 6.8, 3.9 Hz CHOH : m CHCOOEt : m	$\text{COOCH}_2\text{CH}_3\text{-CHF}$	
 2.47	CHF : dddd, $J = 50.9$, 10.4, 8.4, 5.0 Hz CHOH : dddd, $J =$ 11.1, 11.1, 8.3, 4.9 Hz CHCOOEt dddd, $J =$ 11.7, 11.7, 3.8, 3.8 Hz	$\text{CHCO}_2\text{Et-CHOH}$	

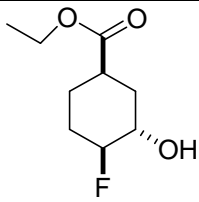
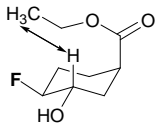
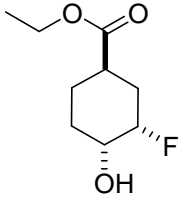
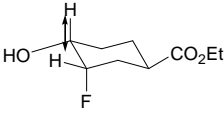
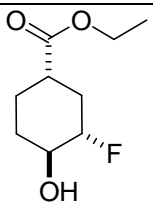
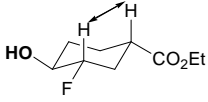
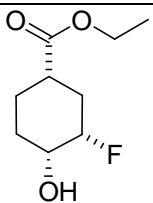
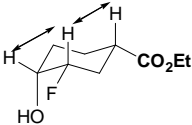
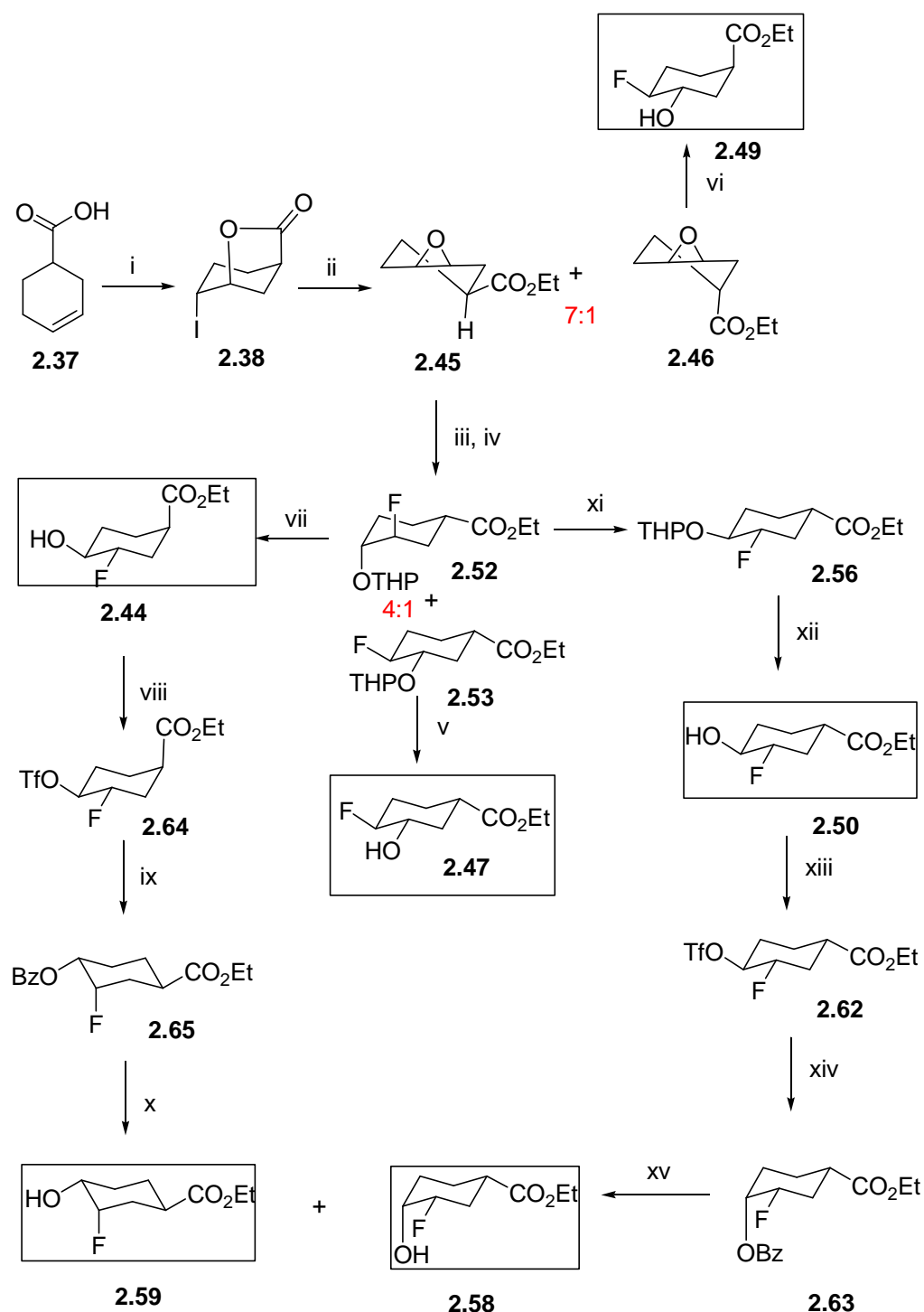
 <p>2.49</p>	$\underline{\text{CHF}}$: dddd, $J = 49.7$, 8.2, 6.9, 3.9 Hz $\underline{\text{CHOH}}$: m $\underline{\text{CHCOOEt}}$: m	$\text{COOCH}_2\underline{\text{CH}}_3\text{-}\underline{\text{CHOH}}$	
 <p>2.59</p>	$\underline{\text{CHF}}$: dddd, $J = 50.2$, 4.5, 2.2, 2.2 Hz $\underline{\text{CHOH}}$: dddd, $J =$ 26.4, 10.9, 4.7, 2.3 Hz $\underline{\text{CHCOOEt}}$: dddd, $J =$ 11.5, 11.5, 3.9, 3.9 Hz	$\underline{\text{CHF}}\text{-}\underline{\text{CHOH}}$	
 <p>2.50</p>	$\underline{\text{CHF}}$: dddd, $J = 50.7$, 11.2, 8.6, 4.7 Hz $\underline{\text{CHOH}}$: m $\underline{\text{CHCOOEt}}$: m	$\underline{\text{CHF}}\text{-}\underline{\text{CHCO}}_2\text{Et}$	
 <p>2.58</p>	$\underline{\text{CHF}}$: dddd, $J = 47.0$, 10.5, 4.8, 2.8 Hz $\underline{\text{CHOH}}$: m $\underline{\text{CHCOOEt}}$: m	$\underline{\text{CHF}}\text{-}\underline{\text{CHOH}}$ $\underline{\text{CHF}}\text{-}\underline{\text{CHCOOEt}}$	

Table 2.13: Stereochemistry of the different fluorohydrins confirmed by proton NMR and NOESY.

All the stereochemistries of the ethyl ester fluorohydrins were confirmed by NOESY NMR. In the case of the isolated methyl ester fluorohydrin **2.40**, no NOESY interactions were observed, but the large coupling constants of the proton adjacent to the fluorine suggest that the fluorine stands in equatorial. Those coupling constants are nearly identical to the ethyl ester equivalent **2.44**. The relative stereochemistry of the methyl ester **2.40** should be identical to the ethyl ester **2.44**, as they were synthesised using identical fluorination steps on epoxides with identical stereochemistry (**Scheme 2.20**). The conformation of both molecules should be identical as they have similar coupling constants for the hydrogen adjacent to the fluorine (**Table 2.13**). Moreover **2.40** was converted in **2.44** after the methyl ester **2.40** was hydrolysed and the resulting acid was esterified to the ethyl ester **2.44** (**Scheme 2.25**).

2.5 Global Synthesis of 6 Different Ethyl Ester Cyclohexane Fluorohydrins.



(i) I_2 , KI, $NaHCO_3$, H_2O , RT, 16 h, 96% (ii) $EtONa$, $EtOH$, THF, $0^\circ C$, 16 h, 48% **2.45**, 7% **2.46** (iii) Et_3N 3HF, RT, 16 h, 76% as a mixture of **2.52** and **2.53** (iv) DHP, H_2NSO_3H , 4 days, 52% **2.52**, 15% **2.53**; (v) *p*-TsOH, $EtOH$, RT, 2 h, 76% (vi) Et_3N :3HF, RT 76% (vii) *p*-TsOH, $EtOH$, RT, 2 h, 99% (viii) Triflic Anhydride, Pyridine, DCM, $0^\circ C$, 2 h, 76% (ix) $Bu_4N^+BzO^-$, toluene, RT, 16 h, 65% (x) Na, $EtOH$, RT, 16 h, 57% **2.59**, 33% **2.58** (xi) *t*-BuOK, toluene, reflux, 40 minutes, 53% (xii) *p*-TsOH, MeOH, RT, 2 h, 92% (xiii) Triflic Anhydride, Pyridine, DCM, $0^\circ C$, 2 h, 67% (xiv) $Bu_4N^+BzO^-$, toluene, RT, 16 h, 61% (xv) Na, $EtOH$, RT, 16 h, 88% **2.58**, 4% **2.59**.

Scheme 2.45: Global optimised synthesis accessing 6 different fluorohydrins.

This synthesis allowed us to access 6 different ethyl ester fluorohydrins, which were fed to *S. hygroscopicus* in order to produce incorporation data. The optimisation of the epimerisation allowed us to obtain a gramme scale synthesis of (1*S**, 3*S**, 4*S**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.50**. A large scale feeding was prepared in order to isolate a quantitative amount of prerapamycin.

3 FEEDING OF THE MOLECULES TO S. HYGROSCOPICUS MG-210.

All the feedings were done at a final concentration of 2 mmolL⁻¹. The incorporation rates were quantified by the presence of the characteristic rapamycin triene detected by UV at 278 nm and quantified by HPLC. Samples were quantified based on a rapamycin calibration curve of absorbance peak area.

3.1 Previous Feedings of 6 Carbon Membered Ring Starter Units.

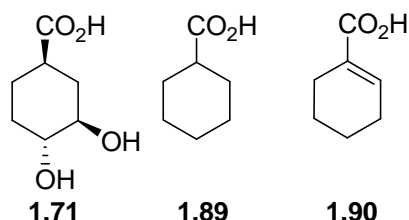


Figure 2.15: Non fluorinated 6 membered rings including a carboxylic acid starter units previously fed.

A series of feeding experiments were attempted by *Biotica Technology Ltd* to *S. hygroscopicus* MG-210 using a broad range of starter units, of these three were 6 carbon membered rings with a carboxylic acid.⁴⁴ One of these was the natural occurring starter unit 3,4-dihydroxycyclohexanecarboxylic acid **1.71** fed as a racemic mixture.⁴⁴ The second was cyclohexanecarboxylic acid **1.89** and the third was cyclohex-1-enecarboxylic acid **1.90**.⁴⁴ All three were successfully incorporated. The corresponding prerapamycins were isolated and characterised by *Biotica Technology Ltd* by mass spectrometry and multidimensional NMR spectroscopy experiments. This allowed them to resolve the structure and stereochemistry of these prerapamycin analogues.⁴⁴ The

calculated incorporation values from the whole broth of the natural occurring starter unit 3,4-dihydroxycyclohexanecarboxylic acid **1.71** and the cyclohexanecarboxylic acid **1.89** were very similar to the naturally occurring production without inhibition of the shikimic acid pathway ($20 - 25 \text{ mgL}^{-1}$)⁹⁴. Incorporation of the cyclohex-1-enecarboxylic acid was about 2 times less ($10 \pm 4 \text{ mgL}^{-1}$). All 3 compounds yielded different prerapamycin analogues (**Table 2.14**). The feeding of the natural occurring starter unit **1.71** yielded the original prerapamycin. The feeding of the cyclohexanecarboxylic acid **1.89** yielded a similar analogue without the presence of the hydroxyl group on carbon 39 of the cyclohexyl ring (39-deshydroxyprerapamycin), and the feeding of cyclohex-1-enecarboxylic acid yielded a similar analogue as the first without the hydroxyl group on carbon 40 (40-deshydroxyprerapamycin)(**Table 2.14**).

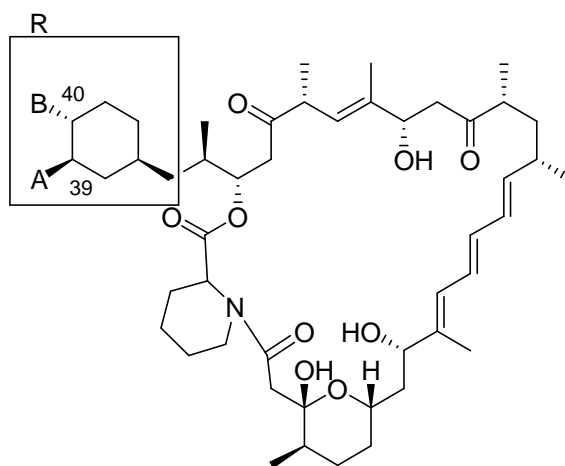


Figure 2.16: Prerapamycin analogues

Starter acid analogue	Prerapamycin analogue (Scheme 1)	Rapamycin analogue produced (mgL^{-1})
 1.71	 R= isolated	20-25 ⁹⁴
 1.89	 R= isolated	20-25
 1.90	 R= isolated	10 ± 4 ⁹⁴

Table 2.14: Previously fed 6 membered ring starter units by *Biotica Technology Ltd.*⁴⁴

These results suggest that hydroxylation can occur on position 3 or 4 of the cyclohexyl moiety depending on the substrate and that the double bond of cyclohex-1-enecarboxylic acid was reduced (**Table 2.14**). *Biotica Technology Ltd* resolved the structures and their stereochemistry using The relative conformation of the cyclohexyl moiety in all 3 isolated prerapamycins suggests that all the groups stand in equatorial positions as a chair conformation (**Table 2.14**). In order to resolve this conformational matter and to synthesise new fluorinated analogues, a range of three monohydroxycyclohexanecarboxylic acid with the hydroxyl group in position 2-, 3- and 4- were fed in parallel with 3-hydroxycyclohex-1-enecarboxylic acid, and the three previously synthesised fluorinated analogues **2.1**, **2.2** and **2.3** (**Table 2.15**).

3.2 Feeding of the Hydroxycyclohexanecarboxylic Acids and Fluorinated Cyclohexanecarboxylic Acids.

Fluorinated compounds synthesised previously (racemic mixtures of both *cis* and *trans* diastereoisomers of 3-fluorocyclohexanecarboxylic acid **2.2** in a 48 : 52 ratio, both *cis* and *trans* isomers of 4-fluorocyclohexanecarboxylic acid **2.1** in a 55 : 45 ratio and 3-fluorocyclohex-1-enecarboxylic acid **2.3** and its methyl ester equivalent **2.17** were fed in parallel with compounds provided by *Biotica Technology Ltd* (cyclohexanecarboxylic acid **1.89**, 4-hydroxycyclohexanecarboxylic acid **1.94**, 3-hydroxycyclohexanecarboxylic acid **1.95**, 2-hydroxycyclohexanecarboxylic acid **1.96**, 3-hydroxycyclohex-1-enecarboxylic acid **1.97**, cyclohex-1-enecarboxylic acid **1.90** and 4,4-difluorocyclohexanecarboxylic acid **2.4** (**Figure 2.17** and **table 2.15**).⁵²

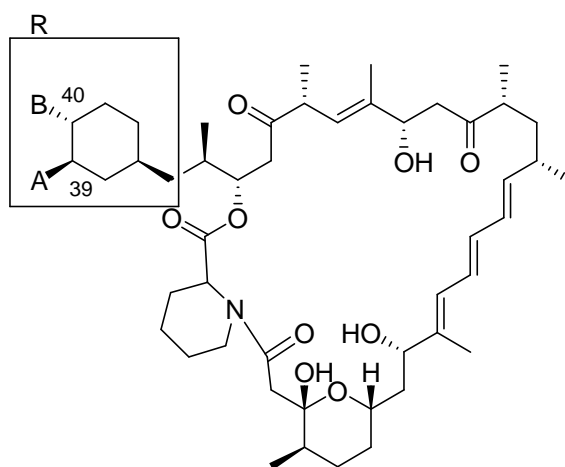


Figure 2.17: Prerapamycin analogues

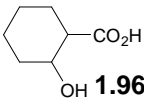
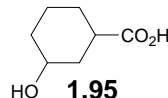
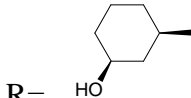
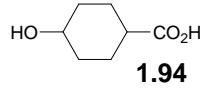
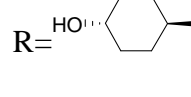
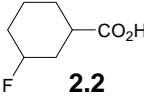
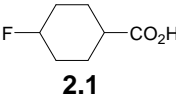
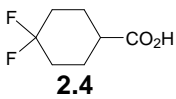
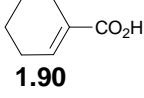
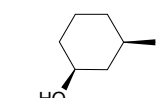
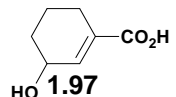
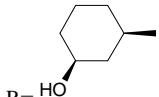
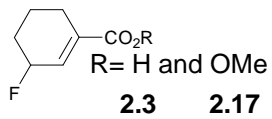
Starter acid analogue	Prerapamycin analogue (Figure 2.17)	Rapamycin analogue produced (mgL ⁻¹)
 1.96		0
 1.95	R= 	3±1
 1.94	R= 	33±3
 2.2		0
 2.1		0
 2.4		0
 1.90	R= 	10±4 ⁹⁴
 1.97	R= 	18±3
 2.3 2.17		0

Table 2.15: Incorporation results of all 6 membered ring cyclohexanoic and cyclohexenoic acids fed to MG-210.

Out of the three hydroxycyclohexanecarboxylic acids **1.94**, **1.95**, **1.96**, 4-hydroxy **1.94** was incorporated in relatively high quantities to yield 39-deshydroxyprerapamycin (Table 2.15), 3-hydroxy **1.95** was incorporated 10 fold less than the previous to yield the 40-deshydroxyprerapamycin analogue, and 2 hydroxy **1.96** was not incorporated. 3-Hydroxycyclohex-1-enecarboxylic acid **1.97** was incorporated in relatively higher

quantities (**Table 2.15**). These results seem to indicate that the starter unit with the hydroxyl group in position 4 **1.94** has a 10 fold incorporation compared to the starter unit with the hydroxyl group in position 3 **1.95** (**Table 2.15**).

Biotica Technology Ltd discovered that the structure and stereochemistry of the prerapamycin analogues obtained for the feeding of **1.95** and **1.97** were both identical to the prerapamycin obtained previously for the feeding of **1.90** (**Table 2.15**). The feeding of **1.94** produced the same prerapamycin analogue as the previous feeding of **1.89** (**Table 2.14 and 2.15**). The structure of isolated prerapamycins⁴⁴ from the feeding of both 3- and 4-hydroxycyclohexanecarboxylic acids suggest that only the all equatorial products in both cases were incorporated (**Table 2.15**).⁵² Those all equatorial molecules are the *trans* 4-hydroxycyclohexanecarboxylic acid and the *cis* 3-hydroxycyclohexanecarboxylic acid (**Figure 2.18**).



Figure 2.18: Suggested all equatorial hydroxycyclohexanecarboxylic acid incorporated

Surprisingly none of the 3 synthesised fluorinated analogues were incorporated, nor was 4,4-difluorocyclohexanecarboxylic acid despite the fact that their hydroxyl equivalents were incorporated. Although the length of the C-F bond is very similar to the C-OH bond, none of the fluorinated analogues were incorporated, suggesting that the hydroxyl group may act as a hydrogen bond acceptor and is required to initiate the biosynthesis on the starter unit.

Hydroxylation that occurred on cyclohex-1-enecarboxylic acid and cyclohex-1-enecarboxylic acid suggest that the presence of the fluorine in position 3 or 4 of the starter unit, or the presence of the hydroxyl group in position 2 seems to obstruct a possible hydroxylation that could occur on the carbon adjacent to the carbon bonded to the fluorine and would allow incorporation and initiation of the biosynthesis.

In order to incorporate a fluorinated starter unit, it seems that a hydroxyl group is necessary in position 3 or 4 and justifies the synthesis and feeding of a series of fluorohydrins instead of monofluorinated compounds. These fluorohydrins, structurally closer to the original starter unit, could give us a better understanding of the stereochemical criteria necessary for a good incorporation.

3.3 Feeding of the 6 fluorohydrins

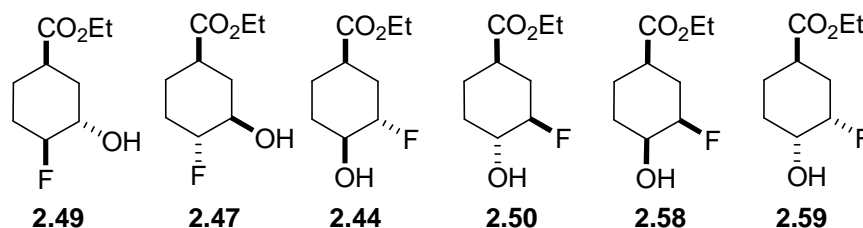

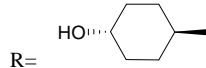

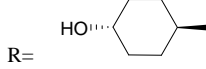


Figure 2.19: Fluorohydrins fed to *S. hygroscopicus*.

Previous feedings of the hydroxycyclohexanecarboxylic acid suggest that the best incorporation is observed for an all equatorial molecule, with the hydroxyl group in position 4. In order to confirm this theory and produce the first fluoroprerapamycins, a range of 6 fluorohydrins were fed as racemic mixtures. Those molecules are all isomers with the fluorine and hydroxyl group in position 3- and 4- and vice versa with various relative stereochemistries. The 6 compounds were all fed in parallel with *trans* 4-hydroxycyclohexanecarboxylic acid **1.94**, cyclohexanecarboxylic acid **1.89** and an unfed control. All the feedings were done in quadruplet, except for compound **2.59** that was fed in triplicate and 4 unfed control tubes were added.

Starter acid analogue	Rapamycin analogue (Scheme 1.)	Corresponding fluorinated prerapamycin MS peaks detected	Rapamycin analogue production (mgL ⁻¹)
None (control)	None	[M+Na] ⁺ 847.7 [M+formate] ⁻ 870.5	0
 1.89	 R=	[M+Na] ⁺ 847.7 [M+formate] ⁻ 870.5	27±2
 1.94	 R=	[M+Na] ⁺ 866.7	29±2

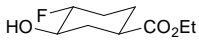
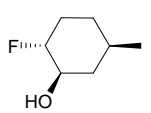
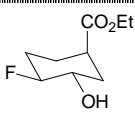
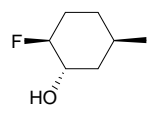
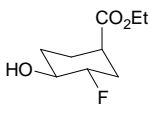
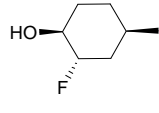
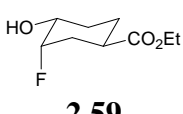
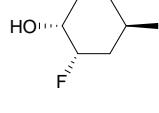
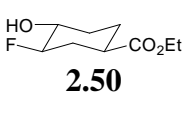
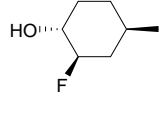
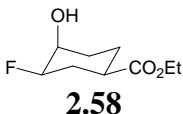
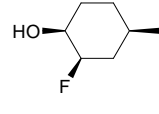
 2.47	 R=	[M+Na] ⁺ 866.7 [M+formate] ⁻ 888.4	5±1
 2.49	 R=	[M+Na] ⁺ 866.7	7±1
 2.44	 R=	[M+Na] ⁺ 866.7 [M+formate] ⁻ 888.4	10±1
 2.59	 R=	[M+Na] ⁺ 866.7 [M+formate] ⁻ 888.5	26±2
 2.50	 R=	[M+Na] ⁺ 866.7 [M+formate] ⁻ 888.6	28±1
 2.58	 R=	[M+Na] ⁺ 866.7	4±1

Table 2.16: Incorporation results of the fluorohydrin feeding.

Surprisingly all 6 fluorohydrins were incorporated, producing the fluorinated analogues (**Table 2.16**). The fluoroprerapamycins were all detected on the LCMS as the [M+Na]⁺ ion (866.7) and in most cases as the [M+formate]⁻ ion (888.4 to 888.6) (See table).

The best incorporations were observed for compound **18** and **2.59**, in which the alcohol group is in position 4, situated in the equatorial position with the ethyl ester group. The best incorporation out of the two being observed for the all equatorial **2.59** compound with the fluorine positioned in the equatorial position 3. This incorporation is similar to the natural starter unit. The worst incorporation was observed when the alcohol group is positioned in axial. Incorporation was fairly low when the fluorine was in position 4.

It was also observed that the incorporations of *trans* 4-hydroxycyclohexanecarboxylic acid and cyclohexanecarboxylic acid were similar to the earlier feedings. Although previously 4-hydroxycyclohexanecarboxylic acid was fed as a 50 : 50 mixture of *cis* and *trans* compound (2 mmolL⁻¹) and therefore half as much *trans* compound (1 mmolL⁻¹)

was fed, the incorporation results were similar. The incorporation of *trans* 4-hydroxycyclohexanecarboxylic acid is very good, and it seems that the production reaches a maximum around 30 mgL⁻¹. The all equatorial compound **2.50** has a very high incorporation close to those 30 mgL⁻¹, and slightly higher than the natural starter unit. It is structurally the closest fluorinated analogue to this starter unit, differing by having a fluorine instead of the hydroxyl group and an ester instead of the acid.

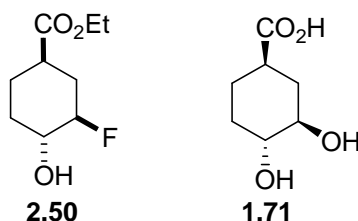


Figure 2.20: Best incorporated fluorohydrin and natural starter unit

The hydroxyl group in the equatorial position is essential to the high incorporation and initiation of the PKS.

As the best incorporated fluorinated starter unit is structurally and conformationally the closest to the natural starter unit, the fluorinated prerapamycin should only differ with the hydroxyl group in position 3 replaced by a fluorine.

In order to isolate and to confirm the structure of the fluorinated prerapamycin a scale up of the feeding was performed.

3.4 Scale up Feeding

912 mg of compound **2.50** in 15 mL of methanol were fed to *S. hygrosopicus*, leading to 16 mg of isolated fluorinated prerapamycin. It appeared clearly as a mixture of two fluorinated prerapamycins that can interconvert. The proton NMR spectrum suggests a ratio of 48:52 for PRERAP1/PRERAP2. The prerapamycins were partially separable (preparative HPLC), but would start interconverting very quickly becoming a mixture again. It is believed that these two compounds are due to the *cis-trans* isomerism on the amide bond (**Figure 2.21**). This phenomenon was observed by for the original

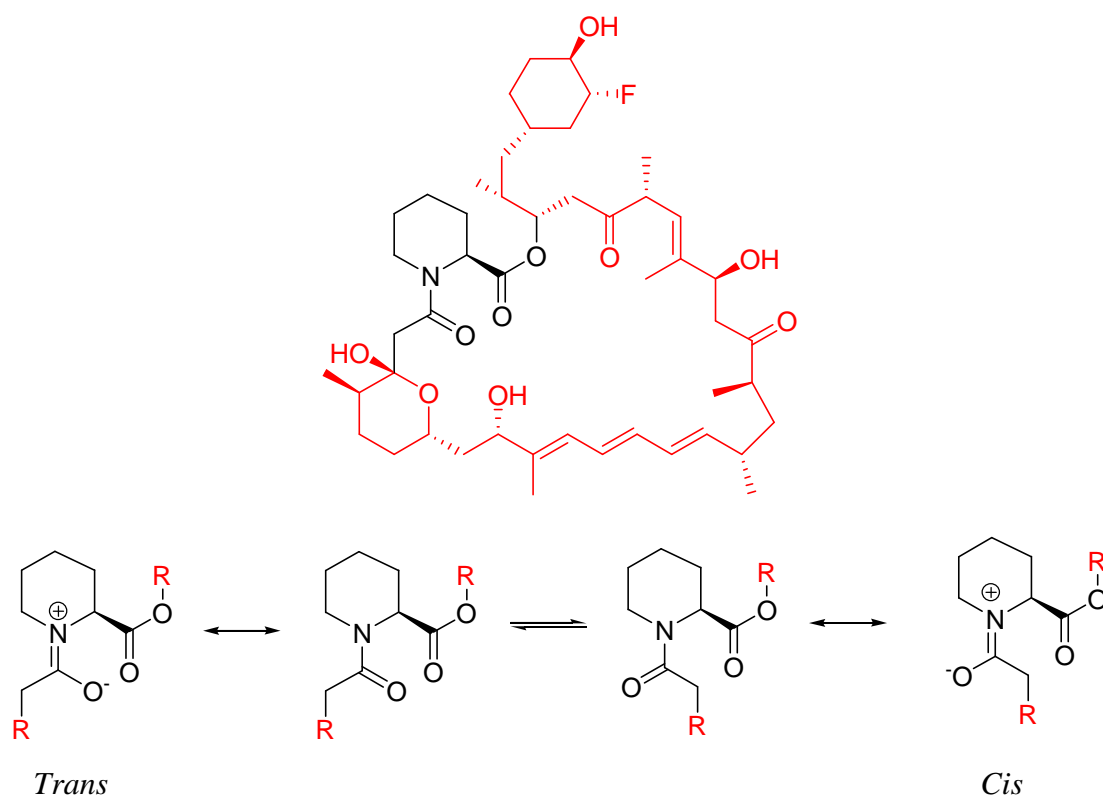


Figure 2.21: *Cis-trans* isomerism on the amide bond.

From the ^1H , ^{13}C , COSY, DEPT, 2D NMR spectra: HMBC, HSQC, COSY and comparing the data from the previous NMR data of non-fluorinated prerapamycin,⁴⁹ the mixture of both isomers was resolved. Both compounds are presented in separate tables (table 2.17 and 2.18). The tables show the assignment for each proton of the corresponding carbon according to the HSQC, the proton-proton correlations of the COSY and the 3 bond hydrogen carbons correlations observed from the HMBC.

The chloroform peak was calibrated on 7.26 ppm in the ^1H NMR spectrum and on 77.0 ppm in the ^{13}C NMR spectrum.

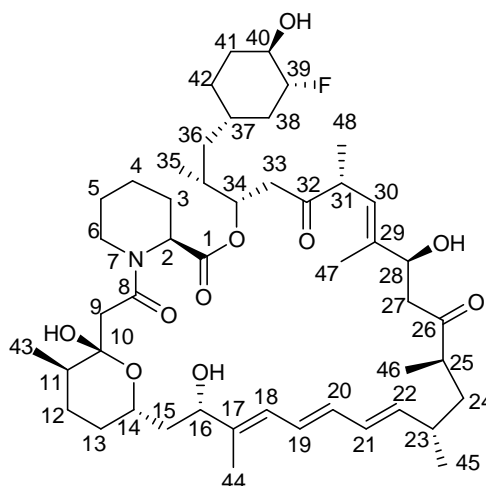


Figure 2.22: Numbered fluorinated prerapamycin analogue.

Position	¹ H-NMR			¹³ C NMR	HMBC
	δ _H / ppm	Multiplicity (Hz)	COSY		
1				171.5	
2	5.38		3a, 3b	52.6	C1, C3, C4
3a	1.78		2, 4a	26.2	C5
3b	2.19		2, 4b		
4a	1.25		3a, 5a	20.9	C2, C6
4b	1.75		3b, 5b		
5a	1.49		4a, 6a, 6b	25.1	C4
5b	1.74		4b, 6a, 6b		
6a	3.25		5a, 5b	44.4	C2, C4, C5
6b	3.84	br.d (13.1)	5a, 5b		
8				172.4	
9a	2.57	d (13.8)	none	40.2	C8, C10, C11
9b	2.89	d (13.7)			
10	none		none	98.6	none
10-OH					
11	1.49		12a, 12b, 43	38.6	C9, C10, C12, C43
12a	1.47		11, 13a	27.6	C10, C11, C13, C14
12b	1.62		11, 13a		
13a	1.34		13a, 14	31.9	C14
13b	1.52		13a, 14		
14	3.89	dddd (11.4, 11.4, 2.0, 2.0)	13a, 13b, 15a, 15b	69.6	C15, C16
15a	1.53		14, 16	40.6	C14, C16, C17
15b	1.68		14, 16		
16	4.08	dd (6.2, 6.2)	15a, 15b	75.6	C14, C15, C17, C18, C44
17	none		None	138.4	
18	6.23	d (12.2)	19	125.1	C16, C17, C19, C20, C44
19	6.33	dd (14.6,	18, 20	127.7	C17, C18, C20,

		11.2)			C21
20	6.19	dd (14.7, 10.7)	19, 21	132.2	C18, C19, C21, C22
21	6.05	dd (14.9, 10.4)	20, 22	130.5	C19, C20, C22, C23
22	5.31	dd (14.9, 9.4 Hz)	21, 23	139.0	C20, C21, C23, C24
23	2.16		22, 24a, 24b, 45	37.3	(C24,C45)
24a	1.30		23, 25	40.3	C22, C23, C25, C26, C45
24b	1.69		23, 25		
25	2.43		24a, 24b, 46	45.6	C23, C24, C26, C46
26				215.5	
27a	2.54	dd (16.7, 3.3)	28	46.4	C26, C28, C29
27b	2.65	dd(16.7, 2.7)	28		
28	4.34		27a, 27b	72.0	C26, C27, C29, C30, C47
29				139.6	
30	5.37	d (9.8 Hz)	31	123.7	C28, C31, C32, C47, C48
31	3.25		30, 48	46.4	C28, C29, C31, C32, C47, C48
32				208.8	
33a	2.61	dd(9.7,18.3)	34	40.6	C32, C34, C35
33b	2.63	m	34		
34	5.13	ddd (6.5, 6.3, 3.8 Hz)	33a, 33b, 35	75.9	C32, C33, C35, C36, C1, C49
35	1.94		34, 36a, 36b, 49	32.6	C49
36a	1.09	dd (3.4, 3.4)	35, 37	37.5	C34, C35, C37, C49
36b	1.20		35		
37	1.41		36a, 38a, 38b, 42a, 42b	32.9	C35, C36, C41
38a	1.46		37, 39	40.7	C36, C40, C42
38b	1.68		37, 39		
39	4.26	dddd (51.3, 10.2, 8.7, 5.0 Hz)	38a, 38b, 40	96.2 d 173.8 Hz	C38, C40
40	3.59		39, 41a, 41b	73.6	C39, C41
41a	1.28		37, 42a, 42b	30.7	C37, C39, C40, C42
41b	1.96		37, 42a, 42b		
42a	1.68		37, 41a, 41b	29.2	C36, C37, C40
42b	0.84		37, 41a,		

			41b		
43	0.95	d (6.5 Hz)	11	16.8	C10, C11, C12
44	1.65	s		12.7	C17, C18
45	1.02	d (6.3 Hz)	23	21.7	C22, C23, C24
46	1.01	d (6.1 Hz)	25	16.6	C24, C25, C26
47	1.59	d (1.0 Hz)		13.1	C28, C29, C30
48	1.08	d (6.8 Hz)	31	16.2	C30, C31, C32
49	0.89	d (6.1 Hz)	35	16.0	C34, C35, C36

Table 2.17: Table presenting ^1H , ^{13}C and 2D correlations from COSY, HSQC, HMBC of PRERAP1.

Position	^1H -NMR			^{13}C NMR	HMBC
	δ_{H} / ppm	Multiplicity (Hz)	COSY		
1				169.0	
2	4.37		3a, 3b	55.7	C1, C3, C4, C8
3a	1.53		2, 4a, 4b	26.7	C1, C5
3b	2.41		2, 4a, 4b		
4a	1.51		3a, 3b, 5a, 5b	20.5	
4b	1.71		3a, 3b, 5a		
5a	1.29		4a, 4b, 6a, 6b	24.4	C6
5b	1.63				
6a	2.15	br.d (13.4)	5a, 5b	39.1	C2
6b	4.47		5a, 5b		
8				171.7	
9a	2.41	d (16.1)		38.8	C8, C10, C11
9b	2.69	d (16.3)			
10				98.4	
10-OH	6.66	br. s			C10, C11
11	1.54		12a, 12b, 43	38.6	C10, C12
12a	1.49		11, 13a, 13b	27.3	C10, C11, C13, C14, C43
12b	1.69		11, 13a, 13b		
13a	1.26		12a, 12b, 14	32.3	C14
13b	1.57		idem		
14	4.22		13a, 13b, 15a, 15b	71.3	C12, C15, C16
15a	1.46		14, 16	40.6	
15b	1.69		14, 16		
16	4.22		15a, 15b	76.0	C14, C15, C17, C18, C44
17	none			141.6	
18	6.09	d (10.5)	19	122.6	C16, C17, C19, C20, C44
19	6.39	dd (14.2, 11.2)	18, 20	128.6	C17, C18, C21

20	6.00	dd (14.2, 10.8)	19, 21	131.2	C18, C21, C22
21	6.06	dd (14.5, 10.6)	20, 22	130.2	C19, C20, C22, C23
22	5.19	dd (14.6, 9.9)	21, 23	139.4	C20, C23, C24, C45
23	2.12		22, 24a, 24b, 45	39.8	C21, C22, C45
24a 24b	1.37 1.98	dd (13.2, 4.0)	23, 25 23, 25	39.7	C22, C23, C25, C26, C46
25	2.44		24a, 24b, 46	46.3	C23, C24, C26, C46
26	none			216.2	
27a 27b	2.45 2.60	dd (17.7, 2.1 Hz)	28 28	48.0	C26, C28, C29
28	4.40		27a, 27b	72.1	C26, C27, C29, C30, C47
29	none			139.7	
30	5.44	d (9.8)	31	125.4	C28, 31, C32, C48
31	3.39	dq (10, 6.8)	30, 48	45.6	C29, C30, C32, C48
32	none			209.0	
33	2.65 2.95	m ddd (18.6, 9.9, 2.5)	34 34	39.7	C32, C34, C35
34	5.36		33a, 33b, 35	74.4	C32, C33, C36
35	1.96		34, 36a, 36b, 49	32.8	C33, C34, C49
36a 36b	1.05 1.16	m dd (7.5, 5.7)	35, 37 35, 37	37.5	C34, C35, C37,
37	1.45		36a, 36b, 38a, 38b, 42a, 42b	33.0	none
38a 38b	1.48 1.66		37, 39 37, 39	40.6	C36, C37, C40
39	4.37-4.22		38a, 38b, 40	96.2 d (173.8Hz)	C40
40	3.61		39, 41a, 41b	73.4	C39, C41
41a 41b	1.29 2.00		40, 42a 40, 42b	30.9	C39, C42
42a 42b	1.73 0.90		37, 41a 37, 41b	29.9	C36, C37, C40, C41,
43	0.95	d (6.5 Hz)	11	16.9	C10, C11, C12
44	1.79	s		15.7	C16
45	1.00	d (6.0 Hz)	23	21.7	C22, C23, C24
46	1.03	d (7.2 Hz)	25	19.1	C24, C25, C26
47	1.49	d (1.1 Hz)		11.8	C28, C29, C30

48	1.01	d (7.1 Hz)	31	14.6	C30, C31, C32
49	0.90	d (6.5 Hz)	35	15.0	C34, C35, C36

Table 2.18: Table presenting ^1H , ^{13}C and 2D correlations from COSY, HSQC, HMBC of PRERAP2.

There is a big difference in chemical shifts observed from one molecule to another in the ^1H NMR spectrum for proton 2 and 6, also observed on the ^{13}C NMR spectrum. This is due to the *cis-trans* isomerism on the amide bond (C8-N7). As carbon 2 and 6 are directly connected to the nitrogen of the amide bond (N7), their chemical environment is directly modified by the *cis-trans* isomerism and so is their chemical shift. The fluorine NMR spectrum confirmed the presence of two fluorinated compounds with two peaks at δ_{F} (282 MHz, CDCl_3) -74.96, -75.85 integrating similar ratios to the ^1H NMR spectrum.

The HRMS also confirms the presence of fluorinated prerapamycins

The calculated exact mass of fluorinated prerapamycin $\text{C}_{48}\text{H}_{74}\text{FNO}_{10}$ is 843.5297

Single Mass Analysis (displaying only valid results)
Tolerance = 5.0 PPM / DBE: min = -5.0, max = 100.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
94 formula(e) evaluated with 1 results within limits (up to 30 best isotopic matches for each mass)
Elements Used:
C: 0-48 H: 0-500 N: 0-1 O: 0-10 F: 0-1 Na: 0-1
Mohammed Nure-Alam - Biotica - 199 SL
MassSpecService2103-1.27 (0.608) Cm (23:34:1.14)

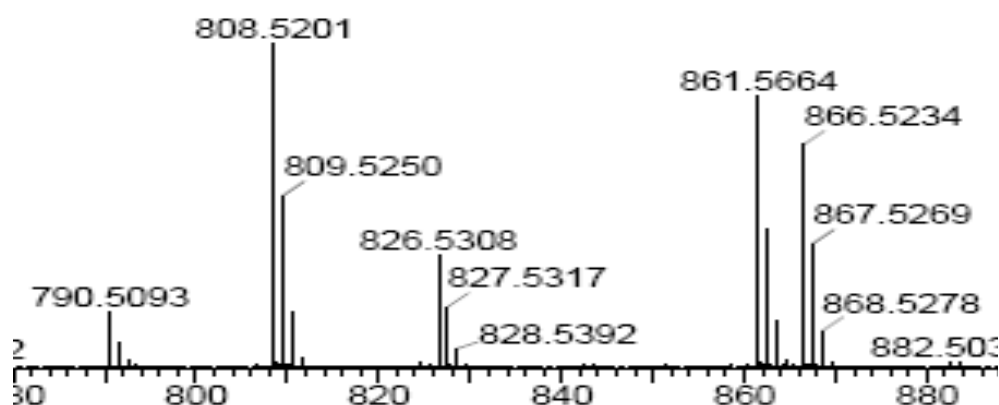
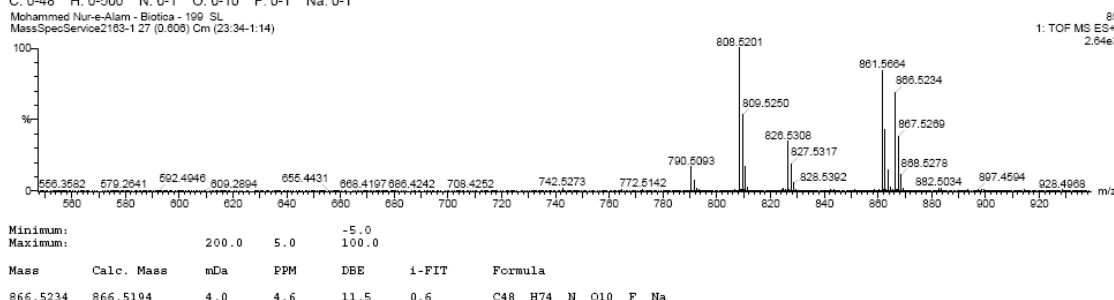


Figure 2.23 HRMS of the mixture of both fluorinated prerapamycin

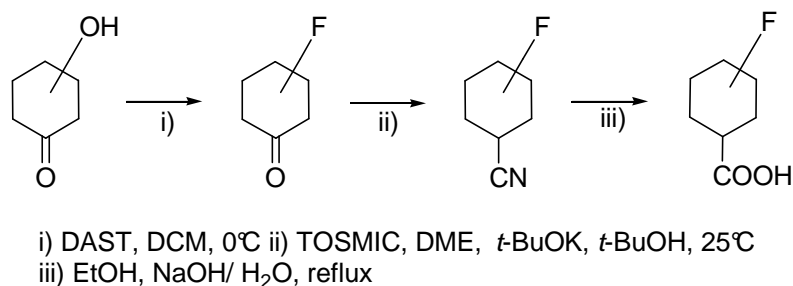
Mass of main peaks	Mass of added or missing fragment	Corresponding fragments	Calculated Mass
866.5234 867.5269 868.5278	+22.9937: Na ⁺	C ₄₈ H ₇₄ FNO ₁₀ + Na ⁺	866.5189
861.5664 861.5664 + 1 861.5664 + 2	+18.0367: H ₂ O	C ₄₈ H ₇₄ FNO ₁₀ +H ₂ O	861.5402
826.5308 827.5317 828.5392	-16.9989: -H ₂ O + H ⁺	C ₄₈ H ₇₄ FNO ₁₀ -H ₂ O +H ⁺	826.5264
808.5201 809.5250 808.5201 + 2	-35.0096: -2H ₂ O + H ⁺	C ₄₈ H ₇₄ FNO ₁₀ -2H ₂ O +H ⁺	808.5158
790.5093 790.5093 + 1 790.5093 + 2	-53.0204: -3H ₂ O + H ⁺	C ₄₈ H ₇₄ FNO ₁₀ -3H ₂ O +H ⁺	790.5053

Table 2.19: Interpretation of the HRMS.

The HRMS showed corresponding masses of C₄₈H₇₄FNO₁₀+Na⁺, C₄₈H₇₄FNO₁₀+ H₂O, C₄₈H₇₄FNO₁₀-H₂O+H⁺, C₄₈H₇₄FNO₁₀-2H₂O+H⁺, C₄₈H₇₄FNO₁₀-3H₂O+H⁺ in the form of 3 main isotopes of decreasing intensity (measured peak intensity for C₄₈H₇₄FNO₁₀+ Na⁺ +0: 100%, +1: 53%, +2: 17%, calculated abundance 100%, 53.5%, 16.1%). The first one of higher intensity only contains the most common isotopes ¹H, ¹²C, ¹⁴N, ¹⁶O and ¹⁹F. The second peak M+1 is a mixture of fluoroprerapamycins containing the most common isotopes (¹H, ¹²C, ¹⁴N, ¹⁶O and ¹⁹F) and one ¹³C or one ²H or one ¹⁷O or one ¹⁵N. ¹⁹F is the only stable isotope of fluorine. The M+2 peak is a mixture of fluoroprerapamycins containing the most common isotopes and two of the previous isotopes (¹³C, ²H, ¹⁷O, ¹⁵N) or one ¹⁸O or one ³H or one ¹⁴C.

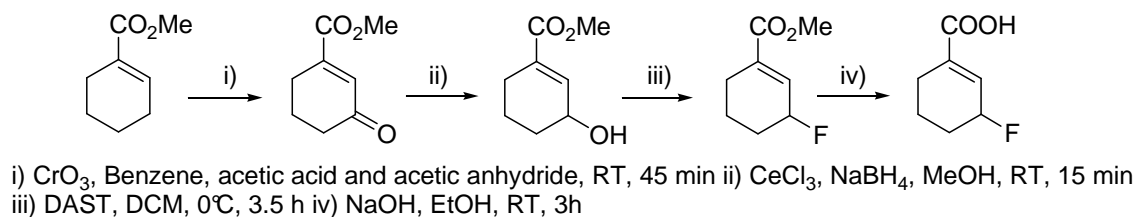
CONCLUSION

4-Fluorocyclohexanecarboxylic acid and 3-fluorocyclohexanecarboxylic acid were synthesised using the same methodology (**Scheme 3.1**). By fluorinating the flattened hydroxyl cyclohexanone intermediate, containing one sp^2 carbon in its 6-membered ring the steric hindrance and dehydration is limited. The yield of the fluorination step was increased in the synthesis toward 4-fluorocyclohexanecarboxylic acid compared to the previous literature method (fluorination of a saturated ring.^{52, 64}).



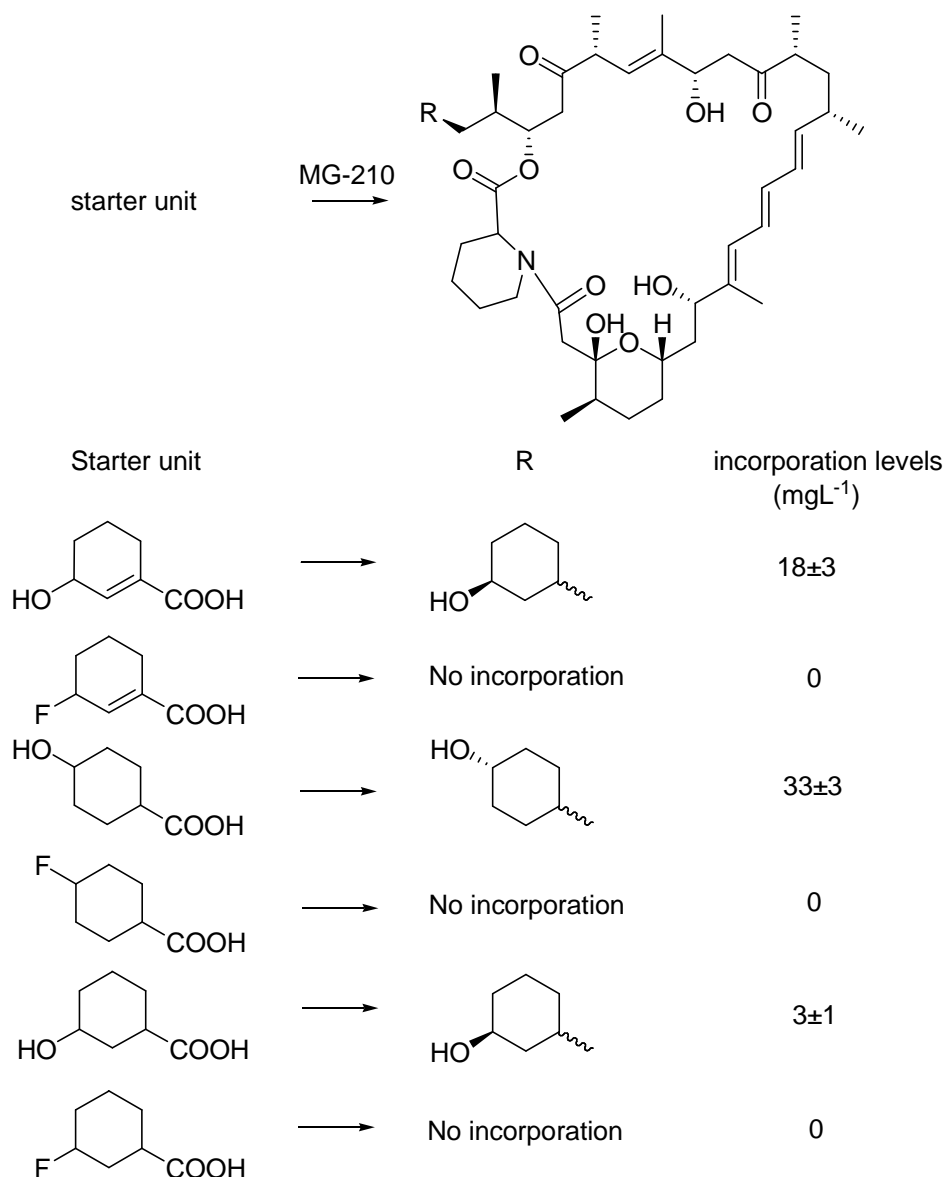
Scheme 3.1: Common synthetic method used to synthesise 4- and 3-fluorocyclohexanecarboxylic acid

3-Fluorocyclohex-1-enecarboxylic acid was synthesised following the literature procedure of Bridge and O'Hagan (**Scheme 3.2**).⁶⁶



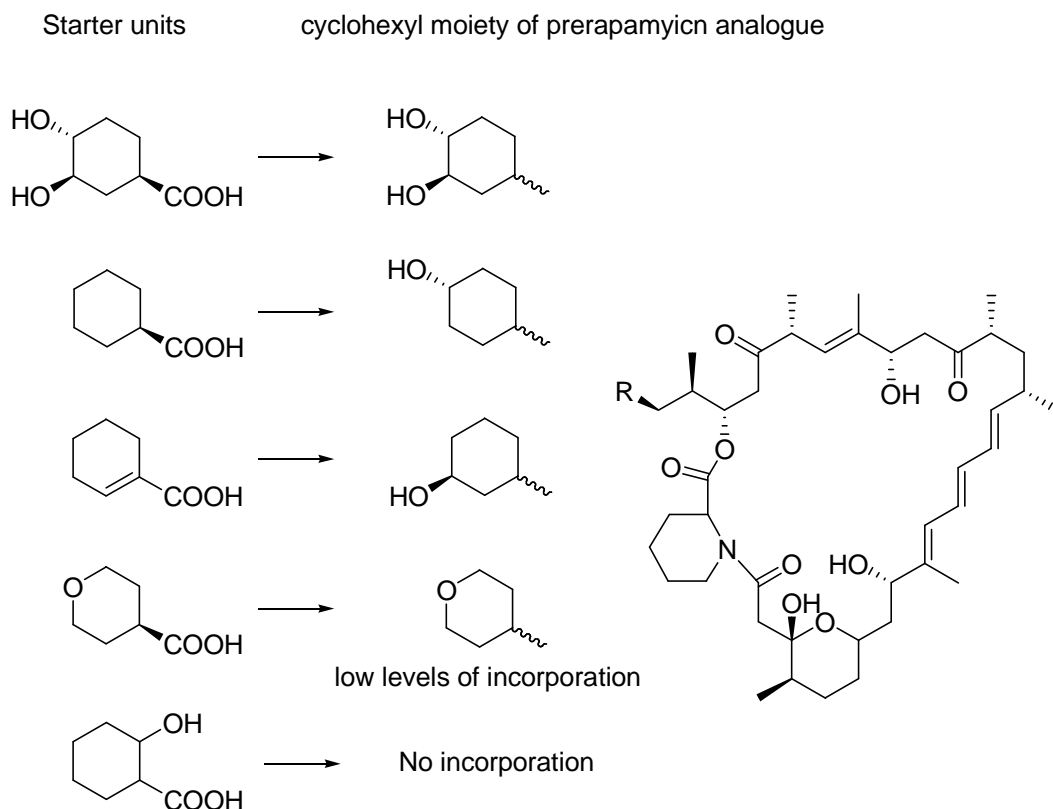
Scheme 3.2: Synthesis of starter unit 3-fluorocyclohexenecarboxylic acid

All three fluorinated compounds: 3-fluorocyclohex-1-enecarboxylic acid, 4 and 3-fluorocyclohexanecarboxylic acid when fed to *S. hygroscopicus* MG-210 were not incorporated to the contrary of the hydroxylated equivalents fed previously by *Biotica Technology Ltd* and fed in parallel with the three fluorinated compounds (**Scheme 3.4**).



Scheme 3.4: Non incorporated monofluorinated starter units and incorporated hydroxyl equivalents.

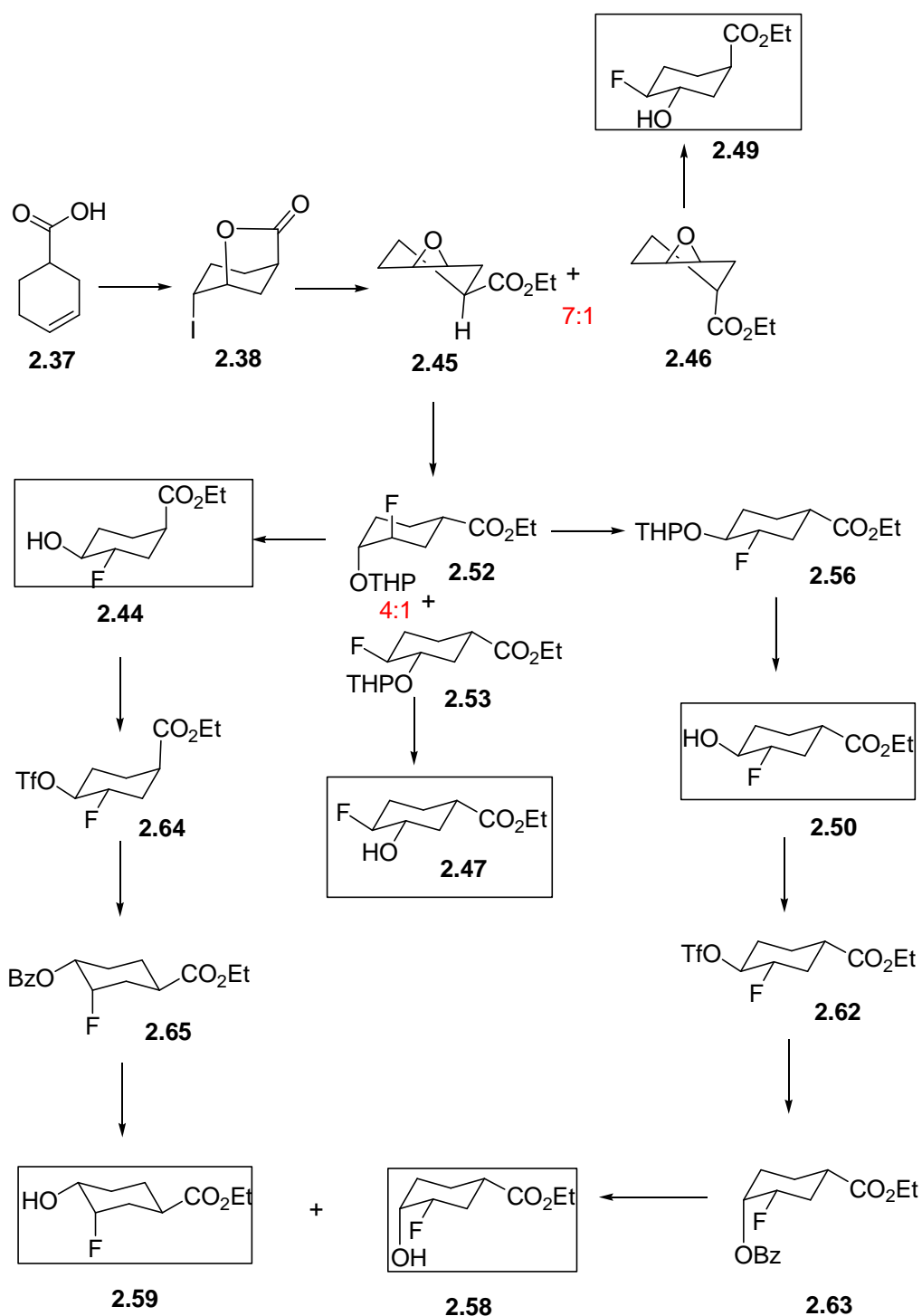
Previous incorporation experiments by *Biotica Technology Ltd* had shown that starter units without a hydroxyl group present had been hydroxylated first by *S. hygroscopicus* MG-210 before being incorporated, with the exception of tetrahydro-2Hpyran-4-carboxylic acid that already contained a hydrogen bond acceptor and was incorporated at very low levels (**Scheme 3.5**). They had also showed that 2-hydroxycyclohexanecarboxylic acid had not been incorporated.



Scheme 3.5: Other 6 membered ring starter units that were fed to *S. hygrosopicus* MG-210 by *Biotica Technology Ltd.*^{44, 52}

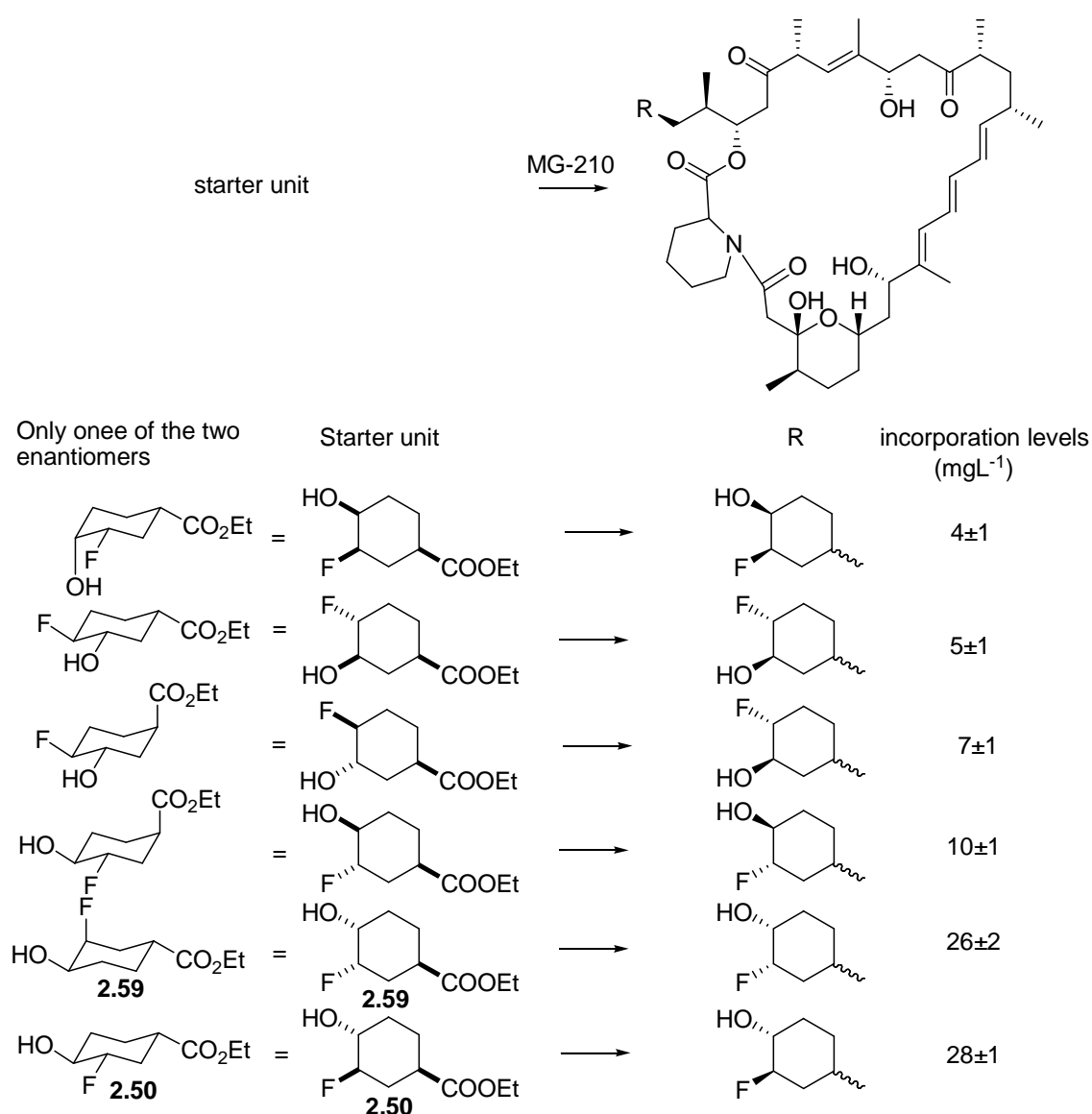
Those results suggest that the presence of a hydrogen bond acceptor and more specifically a hydroxyl group in position 3 or 4 is necessary for incorporation. The presence of fluorine on the ring in position 3 or 4 seems to block any possible enzymatic oxidation in position 3 or 4 necessary for incorporation of a cyclohexane or cyclohexene carboxylic acid derivative. Although the fluorine carbon bond is isosteric to a carbon hydroxyl bond it is not recognised and accepted by the PKS. This could be due to fluorine's different electronic and hydrogen bonding properties compared to a hydroxyl group.⁵²

In order to obtain fluorinated analogues and a better understanding of the rules of incorporations, a range of fluorohydrins containing a hydroxyl group in position 3 or 4 prerequisite for incorporation were synthesised (**Scheme 3.6**).



Scheme 3.6: Synthesis accessing to 6 different fluorohydrins.

The relative stereochemistry of all 6 fluorohydrins was confirmed using NOESY NMR experiments. All 6 fluorohydrins were fed and incorporated by *S. hygroscopicus* MG-210 at various incorporation levels. The highest incorporations were observed for **2.50** and **2.59** when the ethyl ester group and hydroxyl in position 4 were in an equatorial position. The all equatorial product **2.50** had the best incorporation levels out of the two (Scheme 3.7).



Scheme 3.7: Synthesised fluorinated starter units, incorporation levels and corresponding prerapamycin analogues

The feeding of **2.50** was scaled up and the structure of the corresponding prerapamycin analogue resulting from the incorporation of **2.50** was confirmed using ¹H, ¹³C, DEPT, COSY, HSQC, HMBC, NOESY NMR experiments.

Rapamycin analogues could be produced in a similar way using a strain lacking the *Rap K* gene responsible for the synthesis of the starter unit. The activity of the new fluorinated rapamycin analogues could be evaluated and could also give us a better understanding on the importance of the hydrogen bond of the hydroxyl group on carbon 40 with FKBP12, measuring the binding to FKBP12 and FRAP in the ternary complex, and its impact on the biological activity of rapamycin.

**III- EXPERIMENTAL
SECTION
CHAPTER**

General Information and Materials

Reaction glassware was dried in an oven at 120°C and cooled in a dry atmosphere of nitrogen. The solvents used in reactions were dried and distilled prior to use: tetrahydrofuran (THF) and diethyl ether (sodium, benzophenone, under nitrogen), ethyl acetate (calcium chloride, under nitrogen), dichloromethane and diisopropylamine (calcium hydride). Petroleum ether refers to the 40-60°C boiling fraction. Non-aqueous reactions were carried out under an atmosphere of dry nitrogen unless otherwise stated. Air-sensitive reagents and solutions were transferred *via* syringe or cannula and were introduced to the apparatus through rubber septa. Thin layer chromatography was carried out on Merck, Kieselgel 60, F254 glass backed plates. Flash chromatography was carried out using Fluka silica gel-60 (35-70 mm). NMR spectra were recorded on Varian Unity 300 MHz (^1H at 299.908 MHz, ^{13}C at 75.45 MHz), Varian Unity 400 MHz (^1H at 299.956 MHz, ^{13}C at 100.57 MHz) and Bruker Avance BB ATM 500 MHz (^1H at 499.779 MHz, ^{13}C at 125.669 MHz) machines. Samples were run in deuteriochloroform, unless stated otherwise, with chemical shifts reported in parts per million quoted relative to CDCl_3 at $\delta_{\text{H}} = 7.26$, $\delta_{\text{C}} = 77.0$. The deuterated solvent was used as a lock. Coupling constants are reported in hertz. Accurate electrospray ionisation mass spectra (HR ESI-MS) were obtained on a Finnigan MAT 900 XLT mass spectrometer at the EPSRC National Mass Spectrometry Service Centre, Swansea.

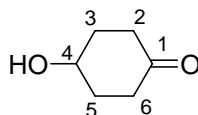
1 SYNTHESIS TOWARDS

MONOFLUOROCYCLOHEXANECARBOXYLIC ACIDS

4,4-DIFLUOROCYCLOHEXANECARBOXYLIC ACID AND 3-FLUOROCYCLOHEXANECARBOXYLIC ACID.

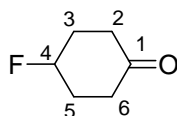
1.1 Synthesis towards 4-Fluorocyclohexanecarboxylic Acid.

General procedure towards the synthesis of 4-hydroxycyclohexanone (2.7)(literature compound)⁶⁷



NaBH₄ (438mg, 11.5 mmol) was added in small portions over a 5 minute period to a solution of 1,4-cyclohexanedione **2.6** (4 g, 35.7 mmol) in methanol (80 mL), stirred under nitrogen at -10°C. The solution was left to stir a further 2.5 hours at -10°C, then slowly neutralised with an aqueous HCl solution (15 mL, 1M). The methanol was evaporated *in vacuo*, and the remaining aqueous solution was extracted in dichloromethane (3×50 mL). The organic extracts were combined and washed with saturated NaCl solution (10 mL), dried over MgSO₄, filtered and evaporated *in vacuo*. The mixture was purified by flash chromatography on silica, eluting with a mixture of Petroleum ether and ethyl acetate (1 : 9), to give the *title compound* **2.7** as a colourless oil (1.45 g; 12.7 mmol, 36%): δ_{H} (300MHz, CDCl₃) 1.84-2.10 (m, 5H, OH, 2H₃, 2H₅), 2.26-2.65 (m, 4H, 2H₂, 2H₆), 4.19 (tt, $J = 3.2, 6.4$ Hz, 1H, H₄); δ_{C} (75 MHz, CDCl₃) 34.1 (C₃, C₅), 37.6 (C₂, C₆), 66.7 (C₄), 211.6 (C₁); MS (CI) m/z 115.1 [M+H]⁺; IR (neat film): $\nu_{\text{max}}(\text{cm}^{-1})$ 3421(OH), 2942 (CH), 1716 (C=O); R_f = 0.26 (100% ethyl acetate)

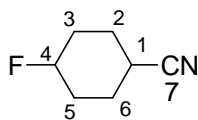
General procedure for the synthesis of 4-fluorocyclohexanone (2.8)⁶⁹



Diethylaminosulphur trifluoride (DAST) (1.05 mL, 7.95 mmol) in dichloromethane (300 mL), cooled to -78°C was added via cannula to a solution of 4-

hydroxycyclohexanone **2.7** (900 mg, 7.89 mmol) in dichloromethane (100 mL) previously cooled to -78°C and stirred under nitrogen. The solution was then placed in an ice bath and left to stir at 0 °C. The reaction went to completion after one hour, before water (100 mL) was added. The organic phase was separated, dried over MgSO₄, filtered and the solvent removed *in vacuo*. The product was purified by flash chromatography on silica, eluting with a mixture of petroleum ether and ethyl acetate (9:1) to give the *title compound* **2.8** as a yellow transparent oil (195 mg, 1.68 mmol, 21%): δ_{H} (300 MHz, CDCl₃) 1.85-2.10 (m, 2H, H_{3eq}, H_{5eq}), 2.26-2.37 (m, 4H, H_{2ax}, H_{6ax}, H_{3eq}, H_{5eq}), 2.59-2.71 (m, 2H, H_{2eq}, H_{6eq}) 4.99 (ttd, $J = 48.5, 5.0, 2.4$ Hz, 1H, H₄); δ_{C} (75 MHz, CDCl₃) 31.0 (d, $J = 21.1$ Hz, C3, C5), 36.2 (d, $J = 4.6$ Hz, C2, C6), 87.2 (d, $J = 169.6$ Hz, C4), 210.0 (C1); δ_{F} (282 MHz, CDCl₃) -187.0; HRMS (EI) m/z 116.0630 (Calcd. 116.0632 for C₆H₉FO); IR (neat film): $\nu_{\text{max}}(\text{cm}^{-1})$ 1716 (C=O); R_f = 0.41 (60: 40 Petrol/ethyl acetate)

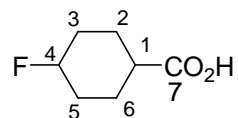
General procedure for the synthesis of 4-fluorocyclohexanecarbonitrile (**2.9**)⁷³



Tosyl methyl isocyanide (55.5 mg, 0.28 mmol) in 1, 1-dimethoxyethane (DME) (0.7 mL) was added to 4-fluorocyclohexanone **2.8** (30 mg, 0.26 mmol) in DME (0.7 mL) stirring under nitrogen before cooling to 0°C. Potassium *tert*-butoxide (58.1 mg, 0.52 mmol) in *tert*-butanol (0.8 mL) and DME (0.4 mL) was added via cannula to the previous cooled mixture. The reaction was left to stir for 3.5 hours at room temperature until completion and ether (30 mL) added. The organic phase was washed with aqueous NaHCO₃ solution (10%, 2×10 mL), saturated aqueous NaCl solution (2×10 mL), dried on MgSO₄, and filtered. The solvents were removed *in vacuo*. The crude product was dissolved in ether (1 mL), filtered through cotton wool and the filtrate evaporated in *vacuo* to give the partially pure *title compound* **2.9** (29.5 mg, 0.23 mmol, 88%) as a light brown oil: δ_{H} (300 MHz, CDCl₃), 1.73-1.85 (m, 4H, CH₂-CF-CH₂), 1.90-2.05 (m, 4H, 2H₂, 2H₆), 2.54-2.65 (m, 0.55 H, H_{1 trans}), 2.72-2.82 (m, 0.45 H, H_{1 cis}), 4.69 (dm(o), $J = 47.9$ Hz), 4.74 (dm(o), 47.9 Hz); δ_{C} (75 MHz, CDCl₃) 24.2 (d, $J = 5.3$ Hz, C2, C6 *cis*), 24.6 (d, $J = 5.3$ Hz, C2, C6 *trans*), 26.5 (C1 *cis*), 26.7 (C1 *trans*), 28.3 (d, $J = 21.0$ Hz, C3, C5 *cis*), 29.1 (d, $J = 21.0$ Hz, C3, C5 *trans*), 87.6 (d, $J = 171.3$ Hz, C4 *trans*), 88.1 (d, $J = 170.5$ Hz, C4 *cis*), 141.8 (C7 *cis*), 152.2 (C7, *trans*); δ_{F} (282 MHz, CDCl₃) -

182.4 (2s); MS (CI) m/z 128.0 $[M+H]^+$; IR (neat film): $\nu_{\max}(\text{cm}^{-1})$ 2233 (-CN); R_f = 0.32 (60: 40 Petrol/ethyl acetate)

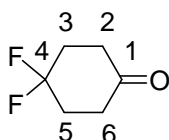
General procedure for the synthesis of 4-fluorocyclohexane carboxylic acid (2.1**)⁷⁴**
(literature compound using a different method)



A solution of aqueous sodium hydroxide (1M, 3.5 mL), and ethanol (0.35 mL) were added to 4-fluorocyclohexanecarbonitrile **2.9** (40.5 mg, 0.32 mmol) in ethanol (0.36 mL), and heated for 4 hours in an oil bath at 82°C. The ethanol was evaporated *in vacuo*. The aqueous phase was washed with diethyl ether (10 mL), acidified between pH 1 and 2 using concentrated hydrochloric acid, then extracted with diethyl ether (3×10 mL). The organic extracts were combined, dried on sodium sulphate, filtered and evaporated to give a 55:45 mixture of the *trans* and *cis* isomer **2.1** (28 mg, 0.192 mmol, 60%) as a light brown resin: δ_{H} (300MHz, CDCl₃) 1.46-2.18 (m, 8H, 2H₂, 2H₃, 2H₅, 2H₆), 2.33-2.345 (m, 1H, H₁), 4.54 (ttd, J = 48.0, 9.5, 3.8 Hz, 0.55H, H_{4ax}), 4.78 (ttd, J = 48.0, 4.6, 2.3 Hz, 0.45, H_{4eq}); δ_{C} (75 MHz, CDCl₃) 22.9 (d, J = 2.9 Hz, C₂, C₆ *trans*) 25.5 (d, J = 10.4 Hz, C₂, C₆ *cis*), 29.8 (d, J = 21.1, Hz, C₃, C₅ *trans*), 30.9 (d, J = 19.7 Hz, C₃, C₅ *cis*), 41.1 (C₁ *trans*), 41.3 (C₁ *cis*), 88.5 (d, J = 169.2 Hz, C₄ *cis*), 90.7 (d, J = 171.8 Hz, C₄ *trans*), 181.1 (C₇ *cis*), 181.2 (C₇ *trans*), δ_{F} (282 MHz, CDCl₃) -174.7, -183.3; MS (ESI) m/z = 147.0 $[M+H]^+$; IR (KBr pellet): $\nu_{\max}(\text{cm}^{-1})$ 2945 (OH), 1701 (C=O).

1.2 Synthesis towards 4,4-Difluorocyclohexanecarboxylic Acid.

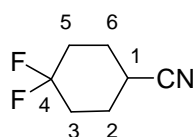
General procedure for the synthesis of 4,4-difluorocyclohexanone (2.10**)⁷⁵**



Diethyl amino sulphur trifluoride (0.1 mL, 0.76 mmol) was added to a solution of 1,4-cyclohexanedione **2.6** (95mg, 0.85 mmol) in dichloromethane (5 mL), cooled at -78°C, and stirred under nitrogen. The mixture was left to warm to room temperature and

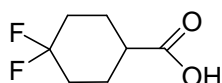
stirred for 24 hours. The solution was then washed with aqueous saturated NaHCO_3 solution (3×3 mL). The organic extract was dried on MgSO_4 , filtered, evaporated *in vacuo* and purified by flash chromatography on neutralised silica eluting with a mixture of petroleum ether and ethyl acetate (8 : 2), to give the *title compound* **2.10** as a yellow oil, with slight impurities (11 mg, 0.08 mmol, 10%): $R_f = 0.48$ (60: 40 Petrol/ethyl acetate); δ_H (300MHz, CDCl_3) 2.31 (tt, $J = 13.3, 7.2$ Hz, 4H, H), 2.55 (t, $J = 7.2$ Hz, 4H); δ_F (300MHz, CDCl_3): -100.5 (s); m/z (relative intensity) 135.1 (M+1).

General procedure for the synthesis of 4,4-difluorocyclohexanecarbonitrile **2.11**⁷³



Tosyl methyl isocyanide (902 mg, 4.6 mmol) in 1, 1-dimethoxyethane (12 mL) was added to a solution of partially purified 4,4-difluorocyclohexanone **2.10** (902 mg, 4.6 mmol) in DME (12 mL) stirred under nitrogen. The mixture was then cooled at 0°C. Potassium *tert*-butoxide (1.014 g, 9 mmol) in *tert*-butanol (6 mL) and DME (6 mL) was added via cannula to the previous cooled mixture. The reaction was left to stir for 3.5 hours at room temperature and was followed by TLC (60:40 Petrol: Ethyl acetate) until completion. Ether (30 mL) was added. The organic extracts were combined and washed successively with NaHCO_3 solution (10%, 2×10 mL), and with saturated NaCl aqueous solution (2×10 mL), then dried on MgSO_4 , and filtered. The solvents were removed under reduced pressure. The crude product was dissolved in ether (1 mL), filtered through cotton wool and the filtrate evaporated *in vacuo* to give the partially purified *title compound* **2.11** (29.5 mg, 0.23 mmol). δ_H (300MHz, CDCl_3) 2.21-1.90 (m, 8H, H2,3,5,6), 2.83-2.68 (m, 1H, H1) m/z (relative intensity) 146.2 (M+1).

General procedure for the synthesis of 4,4-difluorocyclohexanecarboxylic acid (**2.4**)⁷⁴

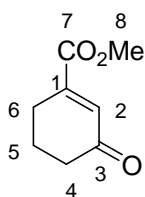


A solution of aqueous sodium hydroxide (1M, 3 mL), and ethanol (0.5 mL) was added to a stirred solution of partially purified 4-fluorocyclohexanecarbonitrile **2.11** (41.5 mg, 0.286 mmol) in ethanol (0.5 mL) and refluxed for 3 hours in an oil bath at 88°C. The ethanol was removed *in vacuo*. The aqueous phase was washed with diethyl ether and

was acidified at 0°C down to pH 2 using concentrated hydrochloric acid, extracted with diethyl ether (3×10 mL), dried over sodium sulphate, filtered and evaporated *in vacuo*. A trace of product **2.4** was detected by GCMS. m/z (relative intensity) 163.2 (M+1).

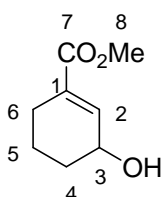
1.3 Synthesis towards 3-Fluorocyclohexenecarboxylic Acid.

General procedure for the synthesis of methyl- 3-oxo-1-cyclohexene-1-carboxylate (2.15) (literature compound).⁷⁶



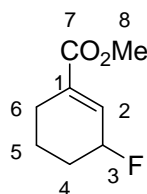
CrO₃ (8.48 g, 84.8 mmol) was added to a stirring solution of acetic anhydride (21.2 mL) and glacial acetic acid (42.4 mL) at 0°C. The previous cooled mixture was added drop wise over a 30 minute period to a stirred solution of methyl-1-cyclohexene-1-carboxylate **2.14** (3.9g, 3.8 mL, 27.8 mmol) in benzene (43 mL), cooled in a water bath. The solution was left to stir another 15 minutes. Benzene was added (50 mL), and the solution was neutralised with a KOH solution (5 molL⁻¹, 85 mL). The solution was the poured in water (180 mL), extracted with diethyl ether (4×300 mL), the organic extracts combined, washed consecutively with a NaHCO₃ solution (3×70 mL) and with a NaCl solution (70 mL), dried over MgSO₄, filtered, and removed *in vacuo*. The product was purified by distillation (0.5 Tor, 55°C) to give the *title compound* **2.15** (2.88g, 18.6 mmol, 67%) as a colourless oil: δ_H (300MHz, CDCl₃) 2.01-2.10 (m, 2H, H5), 2.43-2.47 (m, 2H, H4), 2.56-2.61 (m, 2H, H6), 3.83 (s, 3H, H8), 6.73 (bs, 1H, H2); δ_C (300MHz, CDCl₃) 22.1 (C5), 24.8 (C6), 37.7 (C4), 52.6 (C8), 133.1 (C2), 148.7 (C1), 167.0 (C7), 200.1 (C3); MS (CI) m/z 155.1 [M+H]⁺; IR (neat film): $\nu_{max}(cm^{-1})$ 2951.32 (-OMe), 1713.2 (C=O).

General procedure for the synthesis of methyl- 3-hydroxy-1-cyclohexene-1-carboxylate (2.16)(literature compound).⁷⁷



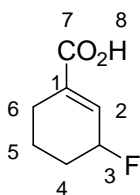
NaBH₄ (332.5 mg, 8.8 mmol) was added to a stirred solution of methyl-3-oxo-1-cyclohexene-1-carboxylate **2.15** (1.34g, 8.7 mmol) and CeCl₃ (H₂O) (3.25g, 8.7 mmol) in methanol (22 mL), and left to stir for 15 minutes. The solution was neutralised to pH 7 with HCl (1molL⁻¹), extracted with diethyl ether (3×50 mL), dried over MgSO₄, filtered, and removed *in vacuo* to give the *title compound* **2.16** (815.5 mg, 5.2 mmol, 60%) as a colourless oil: δ_{H} (300MHz, CDCl₃) 1.61-2.88 (m, 6H, H₆, 4, 5), 3.74 (s, 3H, H₈) 4.30-4.40 (m, 1H, H₃), 6.86-6.88 (m, 1H, H₂); δ_{C} (300MHz, CDCl₃) 19.0 (C₅), 24.2 (C₆), 31.1 (C₄), 51.8 (C₈), 66.0 (C₃), 132.4 (C₂), 139.7 (C₁), 167.7 (C₇); MS (CI) m/z 157.1 [M+H]⁺; IR (neat film): ν_{max} (cm⁻¹) 3417 (-OH), 2945.5 (-OMe), 1716.52 (C=O)

General procedure for the synthesis of methyl-3-fluoro-1-cyclohexene-1-carboxylate (2.17)(literature compound).⁶⁶



DAST (1.15 mL, 1.4g, 8.7 mmol) was added to a solution of Methyl- 3-hydroxy-1-cyclohexene-1-carboxylate **2.16** (1.17 g, 7.49 mmol) in DCM (220 mL) at 0°C stirring under nitrogen. The solution was left to stir for 3.5 hours at room temperature. Water (70 mL) was added to the solution, and the organic phase separated, dried over MgSO₄, filtered, and removed *in vacuo*. The product was purified by flash chromatography on silica, eluting with DCM to give the *title compound* to give the *title compound* **2.17** (1.13 g, 7.15 mmol, 96%) as yellow oil: δ_{H} (300MHz, CDCl₃) 1.61-2.43 (m, 6H, 2H₄, 2H₅, 2H₆) 3.75 (s, 1H, H₈), 5.13 (dm, J_{HF} = 48 Hz, 1H, H₃), 6.87-6.93 (m, 1H, H₂); δ_{C} (300MHz, CDCl₃) 164.8 (C₇), 134.5 (d, J = 19.5 Hz, C₁), 85.7 (d, J = 164.3 Hz, C₃), 52.0 (C₈), 28.3 (d, J = 19.5 Hz, C₄), 24.1 (d, J = 3.0 Hz, C₆), 18.1 (d, J = 4.5 Hz, C₅); δ_{F} (282MHz, CDCl₃): -168.0, -152.1; m/z 159.2 [M+H]⁺; IR (neat film): ν_{max} (cm⁻¹) 2952 (-OMe), 1720 (C=O).

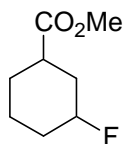
General procedure for the synthesis of 3-fluoro-1-cyclohexene-1-carboxylic acid **2.3 (literature compound).**⁶⁶



Methyl-3-fluoro-1-cyclohexene-1-carboxylate **2.17** (65 mg, 0.41 mmol) was dissolved in ethanol (1 mL) and aqueous NaOH solution (2 mL, 1 molL⁻¹). The mixture was left to stir at room temperature for 3 hours. The aqueous phase was washed with DCM (2×10 mL), then was acidified with an HCl solution (2 molL⁻¹) down to pH 1, extracted with DCM (4×10 mL), the organic extracts were combined and dried over MgSO₄, filtered, and removed *in vacuo*, to give the *title compound* **2.3** (47.4 mg, 0.33 mmol, 80%) as a yellow gel: δ_{H} (300MHz, CDCl₃) 1.45-2.40 (m, 6H, H 6,4,5), 5.15 (dm, $J_{\text{HF}} = 48$ Hz, 1H, H3), 7.03 (d, $J = 9$ Hz, 1H, H2), 8.70 (bs, OH); δ_{C} (75 MHz, CDCl₃) 18.1 (d, $J = 5.2$ Hz, 1C, C5), 23.8 (C6), 28.2 (d, $J = 18.8$ Hz, 1C, C4), 86.1 (d, $J_{\text{HF}} = 165$ Hz, 1C, C3), 136.8 (d, $J = 19.5$ Hz, 1C, C1), 142.0 (s, 1C, C2), 172.1 (C7); δ_{F} (282 MHz, CDCl₃) -172.5; m/z 143.1 (M-1); IR (neat film): ν_{max} (cm⁻¹) 2951.2 (-OH), 1695.9 (C=O).

1.4 Synthesis toward 3-Fluorocyclohexanecarboxylic Acid.

General procedure for the synthesis of methyl-3-fluorocyclohexanecarboxylate (2.18**).**⁷⁸



Hydrogen peroxide (0.4 mL, 27.5%) was added drop by drop to a solution of Methyl-3-fluoro-1-cyclohexene-1-carboxylate **2.17** (58 mg, 0.37 mmol) in ethanol (7 mL) and hydrazine (0.4g, 8 mmol) monohydrate at 0°C.⁷⁸ The solution was left to stir for 4 hours at room temperature. Water was added (2 mL). Then the product was extracted with diethyl ether (3×20 mL) and the organic extracts were combined and dried over Na₂SO₄, filtered, and evaporated *in vacuo*. Some mixture was recovered (30 mg), and a minor peak on the GCMS seemed to indicate the presence of product, but the ¹H NMR spectrum did not confirm that result.

The experiment was repeated with methanol instead of ethanol. No *title compound* was detected, but starting material was recovered.

It was also repeated by adding 2 drops of saturated copper sulphate solution, no *title compound* and no starting material were detected.

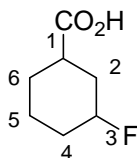
Hydrazine (330.2 mg, 6.596 mmol) acetic acid (1 drop) and saturated copper sulphate solution (1 drop) were added to Methyl-3-fluoro-1-cyclohexene-1-carboxylate **2.17** (10.4mg, 0.066 mmol) in methanol (2.7 mL). Sodium periodate solution (281.0 mg, 1.3 mmol) in water (2.1 mL) was added to the previous solution, drop wise over one hour, maintaining the temperature of the reaction at room temperature.⁷⁸ The solution was then left to stir for 48 hours at room temperature. The methanol was evaporated *in vacuo*. The solution was extracted with ether (3×10 mL), the organic extracts were combined, washed with saturated NaCl solution, and dried over Na₂SO₄, filtered, and evaporated *in vacuo*. No *title compound* was detected.

Acetic acid (0.14 mL) was added drop wise to a solution of methyl-3-fluoro-1-cyclohexene-1-carboxylate **2.17** (20mg, 126.5 µmol) in DMSO (0.85 mL), and dipotassium azodicarboxylate (52 mg, 268 µmol) and was left to stir over night.⁷⁸ The solution was diluted in brine (2 mL), then solution was extracted with pentane (3×5 mL), the organic extracts were combined, dried over Na₂SO₄, filtered, and evaporated *in vacuo*. No *title compound* was detected.

Acetic acid (0.04 mL, 0.715 mmol) in methanol (0.08 mL) was added drop wise over a period of 30 minutes to a stirred solution of methyl-3-fluoro-1-cyclohexene-1-carboxylate **2.17** (21 mg, 0.132 mmol), and dipotassium azodicarboxylate (77 mg, 0.396 mmol) in methanol (0.9 mL) previously cooled to 0°C.⁷⁸ The reaction was left to stir over night. The solution was evaporated, and the residue taken up in water (2 mL) and extracted with DCM (3×10 mL). The organic extracts were combined, washed with saturated bicarbonate solution, dried over Na₂SO₄ and evaporated *in vacuo*. No *title compound* was detected.

All four unsuccessful experiments in order to reduce the double bond were carried out in parallel on 3-fluoro-1-cyclohexene-1-carboxylic acid. No *title compound* was detected.

General procedure for the synthesis of 3-fluorocyclohexanecarboxylic acid (2.2).⁷⁸

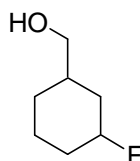


Hydrogen peroxide (0.2 mL, 27.5%) was added drop by drop to a solution of 3-fluoro-1-cyclohexene-1-carboxylic acid **2.3** (27.4 mg, 0.19 mmol) in ethanol (2 mL), hydrazine (165.1 mg, 3.3 mmol) monohydrate and 2 drops of CuSO₄ stirred at 0°C. The solution was left to stir for 6 hours at room temperature. Water was added (1 mL). The aqueous phase was washed with diethyl ether (10 mL), extracted and acidified to pH 1 with HCl solution (2molL⁻¹). Then the product was extracted with diethyl ether (3×10 mL) and the organic extracts were combined and dried over Na₂SO₄, filtered, and evaporated *in vacuo*. No *title compound* was detected

Acetic acid (0.13 mL) was added drop by drop to a solution of 3-fluoro-1-cyclohexene-1-carboxylic acid **2.3** (17 mg, 0.118 mmol) and dipotassium azodicarboxylate (50 mg, 0.258 mmol) in DMSO (0.7 mL). The solution was left to stir for 6 hours. The solution was diluted in brine (2 mL), and extracted with pentane (3×5 mL), the organic extracts were combined, dried over Na₂SO₄, filtered, and evaporated *in vacuo*. No *title compound* was detected.

Acetic acid (0.07 mL, 1.251 mmol) in methanol (0.2 mL) was added drop wise over a period of 30 minutes to a stirred solution of 3-fluoro-1-cyclohexene-1-carboxylic acid **2.3** (25 mg, 0.173 mmol), and dipotassium azodicarboxylate (0.101 mg, 0.521 mmol) in methanol (0.5 mL) previously cooled to 0°C. The reaction was left to stir over night. The solution was evaporated, and the residue taken up in water (2 mL), and extracted with DCM (3×5 mL). The organic extracts were combined, washed with saturated bicarbonate solution, dried and evaporated *in vacuo*. No *title compound* was detected.

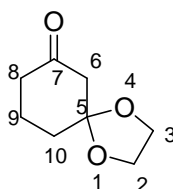
General procedure for the synthesis of (3-fluoro-cyclohexyl)-methanol (2.19)



Methyl-3-fluoro-1-cyclohexene-1-carboxylate **2.17** (96 mg, 0.600 mmol) in THF (0.8 mL) was added to a solution of AlLiH₄ (34 mg, 0.897 mmol) in THF (1.5 mL) at 0°C stirred under nitrogen. The reaction was left to stir for 4.5 hours at 0°C. Water (34μL),

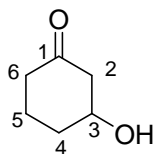
sodium hydroxide solution (34 μ L, 15%), and extra water (102 μ L) were added successively. The mixture was passed through a filtering funnel, and was washed with ether. The filtered solution was then evaporated *in vacuo*, and purified by flash chromatography on silica, eluting with DCM. No *title compound* was detected.

General procedure for the synthesis of 1,4-dioxa-spiro[4.5]decan-7-one (2.21)(literature compound).⁷⁹



p-Toluene sulfonic acid (150 mg,) was added to a solution of ethylene glycol (8.8g, 142 mmol) and cyclohexane-1,3-dione **2.20** (15g, 134 mmol) in benzene(134 mL). The solution was stirred and refluxed for 3.5 hours. The organic solution was left to cool down, and was washed with NaHCO₃ solution (8%, 40 mL). The NaHCO₃ solution was then extracted with benzene (3×40 mL). The organic extracts were combined and added to the previous, and dried over MgSO₄, filtered, evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petrol and ethyl acetate (70:30) to give the *title compound* **2.21** as a yellow oil (6.1g, 39.1 mmol, 29%). δ_{H} (300MHz, CDCl₃) 1.83-1.97 (m, 4H, H9, H10), 2.31-2.35 (m, 2H, H8), 2.59 (s, 2H, H6), 3.93-3.99 (m, 4H, H2, H3); MS (CI) m/z 157.1 (M+1);

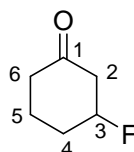
General procedure for the synthesis of 3-hydroxy-cyclohexanone (2.22)(literature compound).⁶⁷



NaBH₄ (400mg, 10.6 mmol) was added in 4 portions during 5 minutes to 1,4-dioxa-spiro[4.5]decan-7-one **2.21** (5.9g, 37.8 mmol) in MeOH (82 mL), cooled at -10°C stirred under nitrogen and was left to stir 1 hour at 0 °C. HCl solution (1 molL⁻¹, 10 mL) was then slowly added to the mixture, to neutralise the solution. The solution was then evaporated *in vacuo*, until all the methanol was evaporated, water was added (10 mL) and was extracted with ethyl acetate (3× 30 mL). The organic extracts were combined, washed with saturated NaCl solution (2× 30 mL), dried over MgSO₄, filtered,

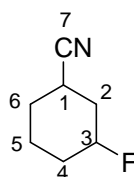
evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petroleum ether and ethyl acetate (55:45) to give the *title compound* **2.22** as a colourless oil (1.05g, 9.2 mmol, 24%): δ_{H} (300MHz, CDCl_3) 1.63-1.83 (m, 3H, H4, 2H5), 1.98-2.13 (m, 2H, H4, OH), 2.32 (t, J = 6.5 Hz, 2H, H6), 2.41 (dd, J = 14.2, 7.7 Hz, 1H, H2), 2.67 (dd, J = 14.5, 4.2 Hz, 1H, H2), 4.19 (tt, J = 7.5, 3.6 Hz, 1H, H3); MS (CI) m/z 114.1 [M];

General procedure for the synthesis of 3-fluoro-cyclohexanone (**2.23**).⁶⁹



Diethyl amino sulphur trifluoride (0.12 mL, 0.91 mmol) was added to a solution of 3-hydroxycyclohexanone **2.22** (100mg, 0.88 mmol) in dichloromethane (35 mL) cooled at -78°C stirred under nitrogen. The mixture was then stirred at 0°C for 4 hours. Water (20 mL) was carefully added. The organic phase was extracted, washed with water (20 mL), dried over MgSO_4 , filtered, evaporated *in vacuo*, and purified by flash chromatography on silica eluting with a mixture of petrol and ethyl acetate (95:5) to give the *title compound* **2.23** as a colourless oil (15 mg, 0.13 mmol, 15%); δ_{H} (300MHz, CDCl_3) 1.76-1.88 (m, 1H, H5), 1.91-2.14 (m, 3H, 2H4, H5), 2.28-2.47 (m, 2H, H6), 2.54-2.74 (m, 2H, H2), 5.06 (dm, J = 49.7 Hz, 1H, H3); HRMS (EI) m/z 116.0630 (Calcd. 116.0632 for $\text{C}_6\text{H}_9\text{FO}$);

General procedure for the synthesis of 3-fluorocyclohexanecarbonitrile (**2.24**).⁷³

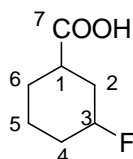


Tosyl methyl isocyanide (392 mg, 1.98 mmol) in 1, 1-dimethoxyethane (DME) (3.3 mL) was added to 3-fluorocyclohexanone **2.23** (205 mg, 1.77 mmol) in DME (3.3 mL) stirring under nitrogen before cooling to 0°C . Potassium *tert*-butoxide (410 mg, 3.67 mmol) in *tert*-butanol (2.9 mL) and DME (2.9 mL) was added via cannula to the previous cooled mixture. The reaction was left to stir for 1.5 hours at room temperature until completion and ether (200 mL) added. The organic phase was washed with aqueous NaHCO_3 solution (10%, 2×50 mL), saturated aqueous NaCl solution (2×50

mL), dried over MgSO_4 , and filtered. The solvents were removed *in vacuo*. The crude product was dissolved in petroleum ether (1 mL), filtered through cotton wool and the filtrate evaporated *in vacuo* to give the partially purified mixture of the *cis* and *trans* title compound **2.24** (73 mg, 0.57 mmol, 32%) as a light brown oil:

δ_{H} (400MHz, CD_3OD) 1.77-1.98 (m, 7H, H2, 2H4, 2H5, 2H6), 2.10-2.30 (m, 1H, H2), 2.81 (tt, $J = 9.44$, 3.65 Hz, 0.52 H, H1trans), 2.99 (tt, $J = 9.59$, 3.71 Hz, 1H, 0.48 H1cis), 4.56 (ttd, $J = 47.7$, 7.70, 3.82 Hz, 0.48H, H3cis), 4.84 (ttd, $J = 47.3$, 5.2, 2.6 Hz, 0.52 H, H3trans); δ_{C} (75 MHz, CD_3OD) 20.4 (d, $J = 4.2$ Hz, C5trans), 21.6 (d, $J = 9.2$ Hz, C1trans), 25.1 (d, $J = 4.6$ Hz, C5cis), 26.3 (d, $J = 10.0$ Hz, C1cis), 29.5 (d, $J = 1.4$ Hz, C6cis), 29.6 (C6trans), 30.9 (d, $J = 20.6$ Hz, C2trans), 32.1 (d, $J = 19.4$ Hz, C2cis), 34.9 (d, $J = 21.4$ Hz, C4trans), 35.5 (d, $J = 21.5$ Hz, C4cis), 88.7 (d, $J = 170.2$ Hz, C3trans), 90.0 (d, $J = 172.6$ Hz, C3cis), 123.0 (C7cis), 123.4 (C7trans); δ_{F} (282 MHz, CDCl_3) -183.9, -173.3; HRMS (ESI) m/z 145.1137 $[\text{M}+\text{NH}_4]^+$ (Calcd. 145.1136 for $\text{C}_7\text{H}_{10}\text{FN}+\text{NH}_4^+$); IR (neat film): $\nu_{\text{max}}(\text{cm}^{-1})$ 2241 (-CN).

General procedure for the synthesis of 3-fluorocyclohexanecarboxylic acid (**2.2**).⁷⁴

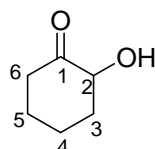


A solution of aqueous sodium hydroxide (1M, 5.2 mL), and ethanol (0.52 mL) was added to 3-fluorocyclohexanecarbonitrile **2.24** (73 mg, 0.57 mmol) in ethanol (0.64 mL), and heated for 3 hours in an oil bath at 82°C. The ethanol was evaporated *in vacuo*. The aqueous phase was washed with diethyl ether (10 mL), acidified to between pH 1 and 2 using concentrated hydrochloric acid, extracted with diethyl ether (3×10 mL), the organic extracts combined, dried on sodium sulphate, filtered and evaporated to give a 6 to 4 mixture of the *trans* and *cis* isomer of the title compound **2.2** (43 mg, 0.29 mmol, 52%) as a yellow oil: δ_{H} (400MHz, CD_3OD) 1.27-1.42 (m, 2H, H5), 1.44-1.55 (m, 1H, H4), 1.56-1.81 (m, 1H, H6), 1.83-1.96 (m, 2H, H2, H4), 1.99-2.17 (m, 1H, H6), 2.24-2.40 (m, 1.52H, H2, 0.52H1), 2.65 (tt, $J = 11.4$, 3.7 Hz, 0.48 H, H1cis), 4.49 (ttd, $J = 48.2$, 10.4, 4.8 Hz, 0.48 H, H3cis), 4.87 (dm, $J = 48.0$ Hz, 0.52 H, H3trans); δ_{C} (75 MHz, CDCl_3) 19.4 (s, $J = 10.1$ Hz, C5trans), 22.2 (d, $J = 11.4$ Hz, C5cis), 27.4 (C6trans), 27.8 (C6cis), 30.1 (d, $J = 21.3$ Hz, C2trans), 31.9 (d, $J = 18.3$ Hz, C2cis), 32.7 (d, $J = 20.9$ Hz, C4cis), 34.5 (d, $J = 20.9$ Hz, C4trans), 37.5 (d, $J = 11.1$ Hz, C1cis), 40.8 (d, $J = 11.0$ Hz, C1trans), 88.3 (d, $J = 168.8$ Hz, C3cis), 90.9 (d, $J = 173.3$ Hz, C3trans), 180.3 (C7cis), 181.5 (C7trans); δ_{F} (282 MHz, CDCl_3) -183.6, -170.4 (d, J

= 47.9 Hz); HRMS (CI)(negative) m/z 145.0668 $[M-H]^-$ (Calcd. 145.0670 for $C_7H_{11}O_2F-H$); IR (neat film): $\nu_{max}(cm^{-1})$ 2943 (OH), 1703 (C=O).

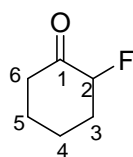
1.5 Synthesis toward 2-Fluorocyclohexanecarboxylic Acid.

General procedure for the synthesis of 2-hydroxycyclohexanone (2.26)(literature procedure).⁸⁰



A mixture of potassium permanganate (8 g, 50.6 mmol), copper sulphate pentahydrate (4 g, 9.2 mmol), *tert*-butanol (2 mL) cyclohexene **2.28** (493 mg, 6 mmol) was stirred for 16 hours. The solution was filtered, removed *in vacuo* to give the *title compound* **2.26** as a colourless oil (146 mg, 1.28 mmol, 21%): δ_H (400 MHz, CD_3OD) 1.66-2.15 (m, 6H), 2.31-2.40 (m, 2H), 2.79 (d, $J = 1.1$ Hz), 4.36 (ddd, 1H, $J = 8.7, 5.2, 1.1$ Hz); δ_C (75 MHz, CD_3OD) 25.5 (C4), 29.5 (C5), 38.7 (C3), 41.8 (C5), 77.4 (C2), 213.9 (C1).

General procedure for the synthesis of 2-fluorocyclohexanone (2.30)(literature compound)⁸¹

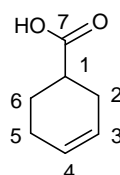


Selectfluor (1.895g, 5.35 mmol) was added to cyclohexanone **2.29** (500 mg, 5.1 mmol) in methanol (50 mL) and refluxed for 3.5 hours. The methanol was removed *in vacuo*, DCM (20 mL) was added, filtered, removed *in vacuo* to give the *title compound* **2.30** as a transparent oil (465 mg, 4.01 mmol, 78%): δ_H (400 MHz, $CDCl_3$) 1.28-2.03 (m, 8H), 4.63 (dm, $J = 49.0$ Hz, 1H, H2)

2 SYNTHESIS OF THE FLUOROHYDRIN CYCLOHEXANECARBOXYLIC METHYL AND ETHYL ESTERS.

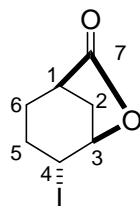
2.1 Synthesis towards 3-Hydroxy-4-fluorocyclohexane carboxylic Methyl Ester.

General procedure for the synthesis of cyclohex-3-enecarboxylic acid (**2.37**)(literature compound).⁸²



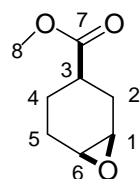
NaOH (3.9 g, 97.5 mmol) was added to a solution of Ag₂O (4.6 g, 19.9 mmol) in water (23 mL). Cyclohex-3-enecarbaldehyde **2.36** (2.16 g, 19.6 mmol) was added in 2 portions, to the previous solution maintained at room temperature, in a water bath and left to stir for 80 minutes. The solution was then filtered through a Buckner funnel. The aqueous solution was washed with ether (2×30 mL), then was acidified with an HCl solution (2 molL⁻¹) down to pH 1 and extracted with diethyl ether (3×50 mL). The organic extracts were combined and dried over MgSO₄, filtered and evaporated *in vacuo*. The product was purified by distillation to give the *title compound* **2.37** (2.06 g, 16.34 mmol, 83%) as a transparent oil: δ_{H} (300MHz, CDCl₃) 1.64-1.77 (m, 1H, H₆), 1.99-2.15 (m, 3H, 2H₅, H₆), 2.25-2.30 (m, 2H, 2H₂), 2.55-2.65 (m, 1H, H₁), 5.64-5.73 (m, 2H, H₃, H₄), 10.76 (bs, 1H, OH); δ_{C} (300MHz, CDCl₃) 24.3 (C₅), 24.8 (C₆), 27.1 (C₂), 39.1 (C₁), 124.9 (C₄), 126.7 (C₃), 182.6 (C₇); MS(EI) *m/z* 127.1 (M+1); IR (neat film): ν_{max} (cm⁻¹) 2953.5 (-OH), 1713.6 (C=O).

General procedure for the synthesis of (1*R, 4*R**, 5*R**)-4-iodo-6-oxa-bicyclo[3.2.1]octan-7-one (2.38)(literature procedure).⁸³**



Cyclohex-3-enecarboxylic acid **2.37** (10 g, 79.3 mmol) was added to a solution of NaHCO₃ in water (425 mL, 0.5molL⁻¹). A solution of iodine (37 g, 145.7 mmol) and potassium iodide (71g, 427.7 mmol) in water (995 mL) was then added to the previous solution and left to stir over night at room temperature. Water (400 mL) and saturated Na₂S₂O₃ solution was added until the solution colour turned white and all the iodine was neutralised (500 mL), then extracted with DCM (4×1L). The organic extracts were combined and washed with NaHCO₃ saturated solution, dried over Na₂SO₄, filtered, and evaporated *in vacuo* to give the title compound **2.38** (19.2g, 96%) as a light yellow powder: δ_{H} (300MHz, CDCl₃) 1.79-1.96 (m, 2H, H6), 2.07-2.16 (m, 1H, H5), 2.34- 2.50 (m, 2H, H3 and H5), 2.66-2.70 (m, 1H, H1), 2.77-2.81 (d, $J = 12.3$ Hz, 1H, H2), 4.48- 4.52 (m, 1H, H4), 4.80-4.84 (t, $J = 5.7$ Hz, 1H, H3); δ_{C} (75 MHz, CDCl₃) 22.9 (C6), 23.7 (C4), 29.5 (C5), 34.3 (C2), 38.4 (C1), 80.0 (C3), 177.7 (C7); MS (CI) m/z 253.1 [M+H]⁺; IR (KBr pellet): ν_{max} (cm⁻¹) 2926 (-O-), 1706 (C=O).

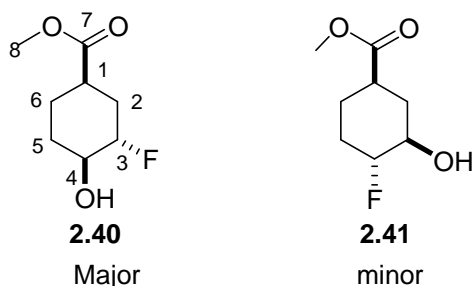
General procedure for the synthesis of 7-oxa-bicyclo[4.1.0]heptane-3-carboxylic acid methyl ester (2.39)(literature compound).⁸³



8-Iodo-2-oxa-bicyclo[3.3.1]nonan-4-one **2.38** (4g, 15.9 mmol) in THF (179 mL) was added to a solution of NaOMe (1 g, 18.6 mmol) in Methanol (179 mL) at 0°C stirring under nitrogen, and was left to stir over night at room temperature. Phosphate ph 7 buffer was added after evaporating the methanol and the THF *in vacuo* until the pH was close to 7 (300 mL), then extracted with diethyl ether. The organic extracts were combined and washed with water and NaCl saturated solution, dried over Na₂SO₄, filtered, and evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petroleum ether and ethyl acetate (9 : 1), to give the *title compound* **2.39** as a colourless oil (0.997g, 6.4 mmol, 40%): δ_{H} (300MHz, CDCl₃) 1.52-1.63 (m,

2H, H4), 1.69-1.79 (m, 1H, H5), 2.12-2.25 (m, 4H, H3, 2H2, H5), 3.12 (bs, 2H, H1, H6), 3.62 (s, 3H, H8); δ_C (75MHz, CDCl₃) 20.9 (C4), 23.9 (C2), 26.2 (C5), 37.7 (C3), 50.6 (C6), 51.7 (C1), 51.7 (C8), 175.2 (C7); m/z (relative intensity) 157.2 (M+1); IR (neat film): $\nu_{\max}(\text{cm}^{-1})$ 2953.3 (C=O), 1732.4 (-OMe).

General procedure for the synthesis of (1*R, 3*S**, 4*S**)-methyl 3-fluoro-4-hydroxycyclohexanecarboxylate⁸⁴ (2.40)**

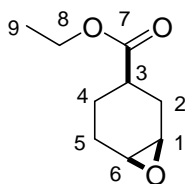


7-Oxa-bicyclo[4.1.0]heptane-3-carboxylic acid methyl ester **2.39** (470 mg, 3.01 mmol) was added to triethylamine trihydrofluoride (4 mL) and was left to stir for 20 hours under nitrogen. NaHCO₃ saturated solution was added until all the HF was neutralised (10 mL) and extracted with ethyl acetate (3×20 mL). The organic extracts were combined and washed with NaHCO₃ saturated solution (50 mL) and NaCl saturated solution (50 mL), dried over Na₂SO₄, filtered, and evaporated *in vacuo*, to give a 4 : 1 mixture of the *title compound* **2.40**, and the isomer **2.41** as a colourless oil (402 mg, 2.28 mmol, 76%). The mixture was purified by flash chromatography on silica eluting with a mixture of DCM and ethyl acetate (95: 5), to give the *title compound* **2.40** as a colourless oil (70 mg, 0.40 mmol, 13%): δ_H (300MHz, CDCl₃) 1.49-1.69 (m, 2H, H5, H6), 1.74-2.03 (m, 3H, H2, H5, H6), 2.09 (bs, 1H, OH), 2.24-2.40 (m, 1H, H2), 2.72-2.79 (m, 1H, H1), 3.73-3.83 (m, 1H, H4), 3.69 (s, 3H, H8), 4.59 (dddd, $J = 49.1, 8.3, 6.9, 3.9$, 1H, H3); δ_C (300MHz, CDCl₃) 23.8 (d, $J = 1.3$ Hz, C6), 28.0 (d, $J = 4.1$ Hz, C5), 29.9 (d, $J = 19.9$ Hz, C2), 38.3 (d, $J = 7.4$ Hz, C1), 51.9 (C8), 70.1 (d, $J = 21.8$ Hz, C4), 92.1 (d, $J = 172.0$ Hz, C3), 174.7 (C7); δ_F (282 MHz, CDCl₃) 186.5 (d, $J = 48.3$ Hz); HRMS (CI) m/z 177.0927 [M+H]⁺ (Calcd. 177.0927 for C₁₈H₁₃FO₃+H⁺); IR (neat film): $\nu_{\max}(\text{cm}^{-1})$ 3603.5(-OH), 2951.5(-OMe), 1731.06 (C=O).

2.2 Synthesis towards Fluorohydrincyclohexanecarboxylic Ethyl Esters and Derivatives.

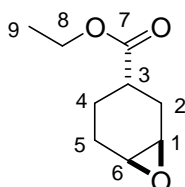
General procedure for the synthesis of (1*R**, 3*R**, 6*S**)-ethyl-7-oxa-bicyclo[4.1.0]heptane-3-carboxylate (**2.45**) and (1*R**, 3*S**, 6*S**)-ethyl-7-oxa-bicyclo[4.1.0]heptane-3-carboxylate (**2.46**)⁸³

(1*R**, 4*R**, 5*R**)-4-iodo-6-oxa-bicyclo[3.2.1]octan-7-one **2.38** (20 g, 79.3 mmol) in THF (810 mL) was added via cannula to a solution of Na (2.3 g, 100 mmol) in ethanol (890 mL) at 0°C stirring under nitrogen, and was left to stir over night at room temperature. Phosphate buffer (pH 7) was added after evaporating the methanol *in vacuo* until the pH gets close to 7 and then extracted with diethyl ether. The organic extracts were combined and washed with water and NaCl saturated solution, dried over Na₂SO₄, filtered, and evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petroleum ether and ethyl acetate (9 : 1), to give the *title compound* **2.45** as a colourless oil (6.5g, 48%), and the *title compound* **2.46** as a colourless oil (0.948 g, 7%).



2.45

2.45: δ_{H} (300 MHz, CDCl₃) 1.24 (t, $J = 7.1$ Hz, 3H, H₉), 1.53-1.67 (m, 2H, H₄, H₄), 1.70-1.84 (m, 1H, H₂), 2.11-2.25 (m, 4H, H₃, H₂, H₅, H₅), 3.15-3.18 (m, 2H, H₁, H₆), 4.11 (q, $J = 7.1$ Hz, 2H, H₈); δ_{C} (75 MHz, CDCl₃) 14.1 (C₉), 20.9 (C₄), 24.0 (C₂), 26.1 (C₅), 37.9 (C₃), 50.7 (C₆), 51.7 (C₁), 60.4 (C₈), 175.0 (C₇); MS (CI) m/z 171.2 [M+H]⁺; IR (neat film): ν_{max} (cm⁻¹) 2987 (-OEt), 1727 (C=O).



2.46

2.46: δ_{H} (400 MHz, CDCl₃) 1.24 (t, $J = 7.1$ Hz, 3H, H₉), 1.36-1.46 (m, 1H, H₄), 1.72-1.80 (m, 1H, H₄), 1.90-1.99 (m, 3H, H₅, H₅, H₂), 2.24-2.29 (m, 1H, H₂), 2.45-2.54 (m, 1H, H₃), 3.13-3.15 (m, 1H, H₆), 3.22-3.24 (m, 1H, H₁), 4.12 (q, $J = 7.1$ Hz, 2H, H₈);

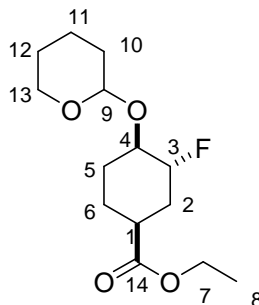
δ_C (75 MHz, $CDCl_3$) 14.1 (C9), 22.6 (C4), 22.7 (C5), 27.0 (C2), 35.7 (C3), 51.3 (C6), 52.1 (C1), 60.3 (C8), 175.6 (C7); HRMS (EI) m/z 170.0936 $[M]^+$ (Calcd. for $C_9H_{14}O_3$ 170.0943); IR (neat film): $\nu_{max}(cm^{-1})$ 2981 (-OEt), 1728 (C=O).

General procedure for the synthesis of (1*S, 3*R**, 4*R**)-ethyl-3-fluoro-4-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate (2.52) and (1*S**, 3*S**, 4*S**)-ethyl-4-fluoro-3-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate (2.53)**^{84, 86}

(1*R**, 3*R**, 6*S**)-ethyl-7-oxa-bicyclo[4.1.0]heptane-3-carboxylate **2.45** (6 g, 35.3 mmol) was added to triethylamine hydrofluoride (18 mL) and was stirring for 40 hours under nitrogen. $NaHCO_3$ saturated solution was added until all the HF was neutralised then extracted in ethyl acetate.⁸⁴ The organic extracts were combined and washed with $NaHCO_3$ saturated solution and NaCl saturated solution, dried over Na_2SO_4 , filtered, evaporated *in vacuo*, purified by flash chromatography on silica eluting with a mixture of DCM and ethyl acetate (90:10), to give a 4 : 1 mixture of the (1*S**, 3*R**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** and (1*R**, 3*R**, 4*R**)-ethyl 4-fluoro-3-hydroxycyclohexanecarboxylate **2.47** as a colourless oil (5.1 g, 26.8 mmol, 76% isolated).

Sulfamic acid (731 mg, 7.5 mmol) was added to a solution of a 4:1 mixture of (1*S**, 3*R**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** and (1*R**, 3*R**, 4*R**)-ethyl 4-fluoro-3-hydroxycyclohexanecarboxylate **2.47** (5.1 g, 26.8 mmol) in 3, 4-dihydro-2H-pyran (9 mL, 8 g, 95.1 mmol), and left to stir under nitrogen for 4 days. The excess 3,4-dihydro-2H-pyran was evaporated *in vacuo*, diethyl ether was added and the solution was filtered, evaporated *in vacuo* and purified by flash chromatography on silica eluting to start with a 100% DCM and then a mixture of DCM and ethyl acetate (98:2) to give (1*S**, 3*R**, 4*R**)-ethyl 3-fluoro-4-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate **2.52** as the major product, as a yellow oil (3.81 g, 13.9 mmol, 52%), and (1*S**, 3*S**, 4*S**)-ethyl 4-fluoro-3-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate **2.53** as the minor product as a yellow oil (1.07 g, 3.9 mmol, 15%) and starting material (1.23 g, 6.5 mmol, 24%) was recovered.

General procedure for the synthesis of (1*S, 3*R**, 4*R**)-ethyl-3-fluoro-4-(tetrahydro-2*H*-pyran-2-yloxy)cyclohexanecarboxylate (2.52):** Reported as two diastereomeric compounds **2.52a** and **2.52b**.

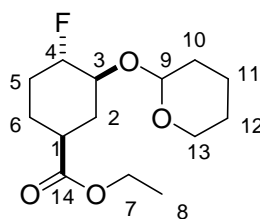


2.52

2.52a: δ_{H} (400 MHz, CDCl_3) 1.19 (t, $J = 7.1$, 3H, H8), 1.42-1.54 (m, 4H, H5, H10, H10, H12), 1.61-1.69 (m, 3H, H5, H6, H11), 1.70-1.90 (m, 4H, H2, H2, H6, H12), 2.03- 2.18 (m, 1H, H2), 2.57-2.64 (m, H1), 3.41-3.46 (m, 1H, H13), 3.60-3.76 (m, 1H, H4), 3.80- 3.85 (m, 1H, H13), 4.10 (q, $J = 7.1$ Hz, 2H, H7), 4.58 (dddd, $J = 47.9$, 6.0, 6.0, 3.4 Hz, 1H, H3), 4.66-4.68 (m, 1H, H9); δ_{C} (100 MHz, CDCl_3) 14.1 (C8), 19.4 (C12), 23.7 (C11), 25.3 (C10), 27.1 (C6), 29.7 (d, $J = 19.9$ Hz, 1C, C2), 30.9 (C5), 37.6 (C1), 60.4 (C7), 62.5 (C13), 73.2 (d, $J = 22.9$ Hz, 1C, C4), 89.9 (d, $J = 173.3$ Hz, 1C, C3), 99.1 (C9), 174.6 (C14); δ_{F} (282 MHz, CDCl_3) -186.83 (d, $J = 50.8$ Hz).

2.52b: δ_{H} (400 MHz, CDCl_3) 1.24 (t, $J = 7.1$, 3H, H8), 1.47-1.59 (m, 4H, H5, H10, H10, H12), 1.66-1.76 (m, 6H, H5, H6, H6, H11, H11, H12), 2.10-2.16 (m, 2H, H2), 2.60-2.67 (m, 1H, H1), 3.45-3.50 (m, 1H, H13), 3.81- 3.88 (m, 2H, H13, H4), 4.11 (q, $J = 7.1$ Hz, 2H, H7), 4.67-4.70 (m(o), 1H, H9), 4.76 (dddd(o), $J = 47.2$, 4.8, 4.8, 2.7 Hz, 1H, H3); δ_{C} (100 MHz, CDCl_3) 14.2 (C8), 19.4 (C12), 22.8 (C11), 24.4 (C6), 25.3 (C10), 29.3 (d, $J = 19.9$ Hz, 1C, C2), 30.9 (C5), 37.3 (C1), 60.4 (C7), 62.4 (C13), 70.8 (d, $J = 26.4$ Hz, 1C, C4), 89.3 (d, $J = 171.4$ Hz, 1C, C3), 96.6 (C9), 175.0 (C14); δ_{F} (282 MHz, CDCl_3) -186.45 (t, $J = 46.0$ Hz, 1C); HRMS (CI) m/z 275.1658 $[\text{M}+\text{H}]^+$ (Calcd. for $\text{C}_{14}\text{H}_{23}\text{FO}_4+\text{H}^+$ 275.1659); IR (neat film): $\nu_{\text{max}}(\text{cm}^{-1})$ 2943 (-OEt), 1732 (C=O).

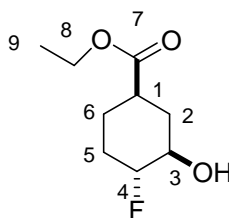
General procedure for the synthesis of (1*S, 3*S**, 4*S**)-ethyl-4-fluoro-3-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate (2.53).**



2.53

2.53: δ_{H} (300MHz, CDCl_3) 1.23-1.29 (t(o), $J = 7.1$ Hz, 3H, H8), 1.44-1.63 (m, 7H), 1.72-1.87 (m, 2H), 1.96-2.04 (m, 1H) 2.13-2.44 (m, 3H), 3.47-3.54 (m, 1H), 3.64-4.03 (m, 2H), 4.12 (q, $J = 7.1$ Hz, 2H), 4.54-4.24 (m, 1H), 4.80-4.96 (m, 1H); δ_{C} (100 MHz, CDCl_3) 14.0, 19.4, 19.5, 22.1, 23.6, 25.3, 25.8, 29.6 (d, $J = 18.9$ Hz), 30.0, 30.5, 30.6, 33.9 (d, $J = 8.4$ Hz), 41.1(d, $J = 1.8$ Hz), 60.5, 61.5, 62.6, 62.8, 73.8 (d, $J = 18.2$ Hz), 77.1 (d, $J = 16.4$ Hz), 95.1 (d, $J = 177.7$ Hz), 100.0 (d, $J = 3.1$ Hz), 174.1; δ_{F} (282 MHz, CDCl_3) -182.1 (d, $J = 50.9$ Hz), -182.6 (d, $J = 50.8$ Hz); HRMS (CI) m/z 275.1658 $[\text{M}+\text{H}]^+$ (Calcd. for $\text{C}_{14}\text{H}_{23}\text{FO}_4+\text{H}^+$ 275.1659); IR (neat film): $\nu_{\text{max}}(\text{cm}^{-1})$ 2944 (-OEt), 1732 (C=O).

General procedure for the synthesis of (1*R, 3*R**, 4*R**)-ethyl-4-fluoro-3-hydroxycyclohexanecarboxylate (2.47)⁸⁷**

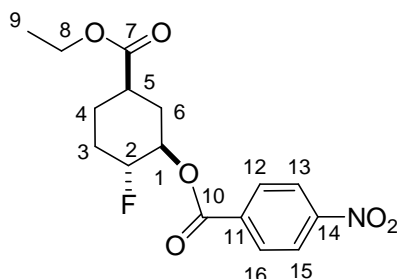


2.47

p-Tosyl sulfonic acid was added in catalytic amount to the solution of (1*S**, 3*S**, 4*S**)-ethyl-4-fluoro-3-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate **2.53** (280 mg, 1.02 mmol) in ethanol (6 mL) and was left to stir for 2 hours. NaHCO_3 saturated solution was added (10 mL) to quench the ethanol was then evaporated *in vacuo*. The solution was extracted with ethyl acetate (3×20 mL) and the organic extracts were combined and washed with NaHCO_3 saturated solution and NaCl saturated solution, dried over Na_2SO_4 , filtered, evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of DCM and ethyl acetate (90: 10) to give **2.47** as a colourless oil (147 mg, 0.77 mmol, 76%).

δ_{H} (400MHz, CDCl_3) 1.25 (t, 3H, $J = 7.1$, H9), 1.49-1.61 (m, 3H, H2, H5, H6), 1.99-2.10 (m, 1H, H6), 2.10-2.20 (m, 1H, H5), 2.22-2.28 (m, 1H, H2), 2.42 (dddd, 1H, H1, $J = 11.7, 11.7, 3.8, 3.8$ Hz), 3.71 (dddd, $J = 11.1, 11.1, 8.3, 4.9$ Hz, 1H, H3), 4.13 (q, $J = 7.1$ Hz, 2H, H8), 4.31 (dddd, $J = 50.9, 10.4, 8.3, 5.0$ Hz 1 H, H4); δ_{C} (75 MHz, CDCl_3) 14.0 (C9), 25.8 (d, $J = 11.2$ Hz, 1C, C6), 28.5 (d, $J = 18.7$ Hz, 1C, C5), 33.5 (d, $J = 7.2$ Hz, 1C, C2), 40.7 (C1), 60.7 (C8), 71.9 (d, $J = 19.2$ Hz, 1C, C3), 95.6 (d, $J = 174.6$ Hz, 1C, C4), 174.3 (C9); δ_{F} (282 MHz, CDCl_3) -186.55; HRMS (ESI) m/z 191.1080 $[\text{M}+\text{H}]^+$ (Calcd. For $\text{C}_9\text{H}_{15}\text{FO}_3+\text{H}^+$ 191.1083); IR (neat film): $\nu_{\text{max}}(\text{cm}^{-1})$ 3446.9 (-OH), 2943.1 (-OEt), 1734.2 (C=O).

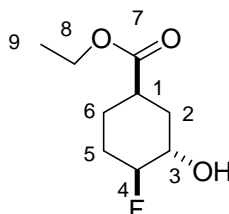
General procedure for the synthesis of (1*R, 2*R**, 5*R**)-5-(ethoxycarbonyl)-2-fluorocyclohexyl 4-nitrobenzoate (2.54)⁸⁵**



4-Nitro-benzoyl chloride (67 mg, 0.36 mmol) was added to 4-fluoro-3-hydroxycyclohexanecarboxylic acid ethyl ester **2.47** (46 mg, 0.24 mmol) in pyridine (3 mL) under nitrogen and left to stir for 2 days. Water (15 mL) was added, and the solution was extracted with ethyl acetate (3×20 mL). The organic extracts were combined and successively washed with oxalic acid solution (10%, 20 mL), cold NaHCO_3 solution (10%, 20 mL), and water (3× 20 mL) and dried over MgSO_4 , filtered, evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petrol and ethyl acetate (95:5) to give the *title compound* **2.54** as a colourless oil (52 mg, 0.15 mmol, 63%): δ_{H} (400MHz, CDCl_3) 1.22 (t, $J = 7.1$ Hz, 3H, H9), 1.60-1.79 (m, 3H, H3, H4, H6), 2.10-2.18 (m, 1H, H3), 2.30-2.36 (m, 1H, H4), 2.43-2.59 (m, 2H, H5, H6), 4.10 (q, $J = 7.11$ Hz, 1H, H8), 4.67 (dddd, $J = 50.4, 10.4, 8.9, 5.0$ Hz, 1H, H2), 5.20 (dddd, $J = 19.6, 11.0, 5.0, 2.2$ Hz, 1H, H1), 8.23 (d, $J = 9.2$ Hz, 2H, H13, H15), 8.30 (d, $J = 8.4$ Hz, 2H, H12, H16); δ_{C} (75 MHz, CDCl_3) 14.0 (C9), 25.6 (d, $J = 10.8$ Hz, C3), 29.3 (s, 1C, C4), 31.5 (s, $J = 6.3$ Hz, 1C, C6), 40.3 (d, 1C, C5), 60.8 (s, 1C, C8), 74.7 (d, $J = 18.7$ Hz, C1), 91.4 (d, $J = 179.9$ Hz, 1C, C2), 123.6 (s, 2C, C13, C15), 130.9 (s, 2C, C12, C16), 135.4 (C11), 150.8 (C14), 164.1 (C10), 173.5 (C7); δ_{F} (300MHz, CDCl_3) 184.9 (d, $J = 53.6$ Hz); HRMS (ESI) m/z 357.1452 $[\text{M}+\text{NH}_4]^+$ (Calcd. 357.1456 for

C₁₆H₁₈FO₆+NH₄⁺); IR (neat film): ν_{max} (cm⁻¹) 2955.6 (-OEt), 2874.4 (-OR), 1722.1 (C=O), 1526.0 (C-NO₂), 1347.6 (C-NO₂), 1607.5, 1526.0, 872.4 (aromatic).

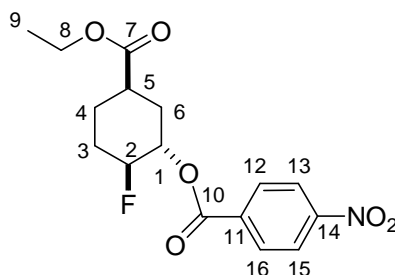
General procedure for the synthesis of (1*R, 3*S**, 4*S**)-ethyl-4-fluoro-3-hydroxycyclohexanecarboxylate (**2.49**)⁸⁴**



(1*R**, 3*S**, 6*S**)-ethyl-7-oxa-bicyclo[4.1.0]heptane-3-carboxylate **2.46** (175 mg, 1.03 mmol) was added to triethylamine trihydrofluoride⁸⁴ (1.5 mL) and was left to stir for 16 hours under nitrogen. NaHCO₃ saturated solution was added until all the HF was neutralised and extracted in ethyl acetate. The organic extracts were combined and washed with NaHCO₃ saturated solution and NaCl saturated solution, dried over Na₂SO₄, filtered, and evaporated *in vacuo*. The mixture was purified by flash chromatography on silica eluting with a mixture of DCM and ethyl acetate (90:10), to give the *title compound* **2.49** as a colourless oil (148 mg, 76%).

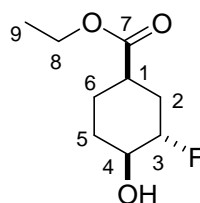
δ_{H} (400 MHz, CDCl₃) 1.26 (t, J = 7.2 Hz, 3H, H₉), 1.60-1.76 (m, 3H, H₂, H₅, H₆), 1.90-2.08 (m, 2H, H₅, H₆), 2.18-2.30 (m(o), 2H, H₂, -OH), 2.69-2.72 (m, 1H, H₁), 3.91-4.00 (m, 1H, H₃), 4.15 (q, J = 7.1 Hz, 2H, H₈), 4.38 (dddd, J = 49.7, 8.2, 6.9, 3.9 Hz, 1H, H₄); δ_{C} (75 MHz, CDCl₃) 14.1 (C₉), 23.8 (d, J = 7.4 Hz, 1C, C₆), 26.3 (d, J = 19.1 Hz, 1C, C₅), 31.7 (d, J = 3.8 Hz, 1C, C₂), 37.6 (C₁), 60.6 (C₈), 68.8 (d, J = 22.5 Hz, 1C, C₃), 93.4 (d, J = 173.5 Hz, 1C, C₄), 174.6 (C₇); δ_{F} (282 MHz, CDCl₃) -186.3; HRMS (ESI) m/z 208.1345 [M+NH₄]⁺ (Calcd. for C₉H₁₅FO₃+NH₄⁺ 208.1343); IR (neat film): ν_{max} (cm⁻¹) 3439.3 (-OH), 2940.4 (-OEt), 1727.2 (C=O).

General procedure for the synthesis of (1*S,2*S**,5*R**)-5-(ethoxycarbonyl)-2-fluorocyclohexyl 4-nitrobenzoate (**2.51**)⁸⁵**



4-Nitro-benzoyl chloride (135 mg, 0.73 mmol) was added to 3-fluoro-4-hydroxycyclohexanecarboxylic acid ethyl ester **2.49** (70 mg, 0.37 mmol) in pyridine (2 mL) under nitrogen and was left to stir over night. Water (6 mL) was added, and the solution was extracted with ethyl acetate (3×20 mL). The organic extracts were combined and successively washed with oxalic acid solution (10%, 20 mL), cold NaHCO₃ solution (10%, 20 mL), and water (2× 20 mL) and dried over Na₂SO₄, filtered, evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petrol and ethyl acetate (95:5) to give the *title compound* **2.51** as white crystals (76 mg, 0.22 mmol, 59%) δ_{H} (300 MHz, CDCl₃) 1.26 (t, J = 7.1 Hz, 3H, H9), 1.76-2.02 (m, 5H, 2H3, 2H4, H6), 2.23-2.34 (m, 1H, H6), 2.67-2.77 (m, 1H, H5), 4.16 (q, J = 7.1 Hz, 2H, H8), 4.70 (dddd, J = 47.9, 5.6, 5.3, 2.9 Hz, 1H, H2), 5.36-5.44 (m, 1H, H1), 8.18 (d, J = 9.1 Hz, 2H, H13, H15), 8.29 (d, J = 9.1 Hz, 2H, H12, H16); δ_{C} (75 MHz, CDCl₃) 14.1 (C9), 23.0 (d, J = 4.5 Hz, C4), 26.6 (d, J = 20.0 Hz, C3), 28.7 (C6), 37.7 (C5), 60.8 (C8), 70.9 (d, J = 27.4 Hz, C1), 87.9 (d, J = 174.7 Hz, 1C, C2), 123.7 (s, 2C, C13, C15), 130.9 (s, 2C, C12, C16), 135.4 (C11), 150.9 (C14), 163.6 (C10), 174.1 (C7); δ_{F} (282 MHz, CDCl₃) -188.4; HRMS (ESI) m/z 357.1454 [M+NH₄]⁺ (Calcd. 357.1456 for C₁₆H₁₈FO₆+NH₄⁺); IR (neat film): ν_{max} (cm⁻¹) 2952.6 (-OEt), 2917.3 (-OR), 1724.8 (C=O), 1522.6 (C-NO₂), 1348.6 (C-NO₂), 1607.6, 1522.6, 868.2 (aromatic); X-ray structure available.

General procedure for the synthesis of (1*S, 3*R**, 4*R**)-ethyl-3-fluoro-4-hydroxycyclohexanecarboxylate (**2.44**)⁸⁴**



(1*R**, 3*R**, 6*S**)-ethyl-7-oxa-bicyclo[4.1.0]heptane-3-carboxylate **2.45** (70 mg, 0.41 mmol) was added to triethylamine trihydrofluoride (1 mL) and was left to stir for 40 hours under nitrogen. NaHCO₃ saturated solution was added until all the HF was neutralised then extracted in ethyl acetate. The organic extracts were combined and washed with NaHCO₃ saturated solution and NaCl saturated solution, dried over MgSO₄, filtered, evaporated *in vacuo*, purified by flash chromatography on silica eluting with a mixture of DCM and ethyl acetate (95:5), to give the *title compound* **2.44** (8 mg, 0.042 mmol, 10%).

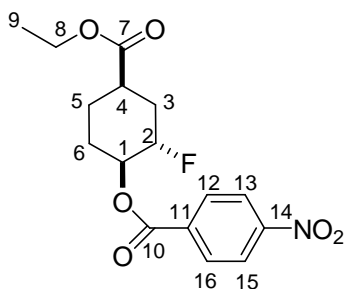
A sodium hydroxide aqueous solution (2 mL, 1 molL⁻¹) was added to (1*R*,3*S*,4*S*)-methyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.40** (25.4 mg, 0.144 mmol), and was left to stir for 3 hours. The aqueous solution was washed with ethyl acetate (2 mL), extracted, acidified to pH 1 with dilute HCl aqueous solution (0.1 molL⁻¹), extracted with ethyl acetate (3×10 mL). The organic extracts were combined and washed with NaCl saturated solution, dried over Na₂SO₄, filtered and evaporated *in vacuo* to the crude acid **2.43** (17.3 mg). Concentrated sulfuric acid (2 drops) was added to the crude acid in dry ethanol (3 mL) and was left to stir for 20 hours. The reaction was neutralised with NaHCO₃ saturated solution (3 mL). The ethanol was evaporated *in vacuo*. The aqueous solution was extracted with ethyl acetate (4×10 mL). The organic extracts were combined, washed with NaHCO₃ saturated solution and NaCl saturated solution, dried over Na₂SO₄, filtered, evaporated *in vacuo*, purified by flash chromatography on silica eluting with a mixture of petroleum ether and ethyl acetate (80 : 20), to give the *title compound* **2.44** (12 mg, 0.063 mmol, 44%).

A catalytic amount of p-Toluene sulfonic acid was added to the solution of (1*S**, 3*R**, 4*R**)-ethyl 3-fluoro-4-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate **2.52** (310 mg, 1.13 mmol) in ethanol (8 mL) and was left to stir for 2 hours.⁸⁷ NaHCO₃ saturated solution was added (10 mL) to quench, the ethanol was then evaporated *in vacuo*. The solution was extracted with ethyl acetate (3×20 mL) and the organic extracts were combined and washed with NaHCO₃ saturated solution and NaCl saturated solution, dried over Na₂SO₄, filtered, evaporated *in vacuo* and purified by flash chromatography

on silica eluting with a mixture of DCM and ethyl acetate (90: 10) to give **2.44** as a colourless oil (212 mg, 99%).

δ_{H} (300MHz, CDCl_3) 1.24 (t, $J = 7.1$ Hz, 3H, H9), 1.47-1.67 (m, 2H, H5, H6), 1.72-2.02 (m, 3H, H2, H5, H6), 2.21-2.36 (m, 2H, H2, -OH), 2.68-2.75 (m, 1H, H1), 3.71-3.80 (m, 1H, H4), 4.12 (q, $J = 7.1$ Hz, 2H, H8), 4.58 (dddd, $J = 49.1, 8.1, 6.8, 3.9$ Hz, 1H, H3); δ_{C} (75 MHz, CDCl_3) 14.0 (C9), 23.7 (C6), 27.9 (d, $J = 3.9$ Hz, 1C, C5), 29.7 (d, $J = 20.0$ Hz, 1C, C2), 38.2 (d, $J = 7.3$ Hz, 1C, C1), 60.6 (C8), 70.0 (d, $J = 22.5$ Hz, 1C, C4), 92.2 (d, $J = 172.3$ Hz, 1C, C3), 174.5 (C7); δ_{F} (282 MHz, CDCl_3) -186.5; HRMS (FI) m/z 191.1083 $[\text{M}+\text{H}]^+$ (Calcd. for $\text{C}_9\text{H}_{15}\text{FO}_3+\text{H}^+$ 191.1083); IR (neat film): $\nu_{\text{max}}(\text{cm}^{-1})$ 3424(-OH), 2940 (-OEt), 1727 (C=O).

General procedure for the synthesis of (1*S, 2*S**, 4*R**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl 4-nitrobenzoate (**2.48**)⁸⁵**

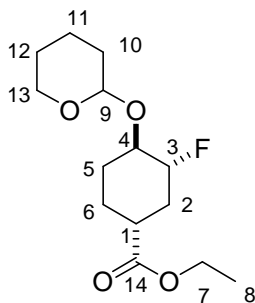


4-Nitro-benzoyl chloride (187 mg, 1.01 mmol) was added to 3-fluoro-4-hydroxycyclohexanecarboxylic acid ethyl ester **2.44** (156 mg, 0.82 mmol) in pyridine (1.2 mL) under nitrogen and was left to stir over night. Water (6 mL) was added, and the solution was extracted with ethyl acetate (3×20 mL). The organic extracts were combined and successively washed with oxalic acid solution (10%, 20 mL), cold NaHCO_3 solution (10%, 20 mL), and water (2× 20 mL) and dried over Na_2SO_4 , filtered, evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petrol and ethyl acetate (95:5) to give the *title compound* **2.48** as a colourless oil (174 mg, 0.39 mmol, 63%), that crystallised after 2 weeks at -20°C :

δ_{H} (300 MHz, CDCl_3) 1.29 (t, $J = 7.1$ Hz, 3H, H9), 1.82-2.36 (m, 6H, H3, H5, H6), 2.82 (tt, $J = 8.3, 4.3$ Hz, 1H, H4), 4.18 (q, $J = 7.1$, 2H, H7), 4.90 (dddd, $J = 47.4, 6.4, 6.1, 3.5$ Hz, 1H, H2), 5.20-5.28 (m, 1H, H1), 8.21 (d, $J = 8.6$ Hz, 2H, H13, H15), 8.30 (d, $J = 8.6$ Hz, 2H, H12, H16); δ_{C} (75 MHz, CDCl_3) 14.2 (C8) 23.7 (C5), 25.4 (C6), 30.2 (d, $J = 19.5$ Hz, C3), 37.5 (d, $J = 5.2$ Hz, C4) 60.8 (C7), 71.9 (d, $J = 26.3$ Hz, C1), 87.8 (d, $J = 174.0$ Hz, C2), 123.6 (s, 2C, C13, C15), 130.8 (s, 2C, C12, C16), 135.2 (s, 1C, C11), 150.7 (s, 1C, C14), 163.7 (C10), 174.3 (C9); δ_{F} (282 MHz, CDCl_3) -186.8 (d, $J = 29.6$

Hz); HRMS (FI) m/z 340.1196 $[M+H]^+$ (Calcd. 340.1196 for $C_{16}H_{18}FO_6+H^+$); IR (neat film): $\nu_{max}(cm^{-1})$ 2952.6 (-OEt), 1724.8 (C=O), 1522.6. (C-NO₂), 1348.6 (C-NO₂), 1607.6, 1522.6, 868.1 (aromatic); X-ray data available.

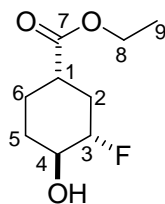
General procedure for the synthesis of (1*R, 3*R**, 4*R**)-ethyl-3-fluoro-4-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate (2.56)**



Potassium *tert*-butoxide (186 mg, 1.65 mmol) was added to a racemic mixture of (1*S**, 3*R**, 4*R**)-ethyl-3-fluoro-4-(tetrahydro-2H-pyran-2-yloxy) cyclohexanecarboxylate **2.52** (2.20g, 8.02 mmol) in freshly distilled toluene (104 mL) under nitrogen and refluxed for 40 minutes. The reaction vessel was then allowed to cool to room temperature under nitrogen. The solution was then purified by flash chromatography on silica eluting with hexane, then a mixture of hexane and ethyl acetate (95:5), to give the *title compound* **2.56** as a yellow oil (1.17g, 53%) and starting material (1.01g, 46% recovered).

δ_H (300 MHz, $CDCl_3$) 1.23 (t, $J = 7.1$ Hz, 3H), 1.33-1.86 (m, 9H), 1.94-1.98 (m, 1H), 2.11-2.15 (m, 1H), 2.31-2.36 (m, 2H), 3.48-3.53 (m, 1H), 3.63-3.75 (m, 1H), 3.87-4.02 (m, 1H), 4.12 (t, $J = 7.1$ Hz, 2H), 4.29-4.47 (m, 1H), 4.80-4.83 (m, 1H); δ_C (75 MHz, $CDCl_3$) 14.1, 19.7, 25.3, 26.7 (d, $J = 1.78$ Hz), 30.3 (d, $J = 8.17$ Hz), 30.6, 30.8, 33.2 (d, $J = 20.30$ Hz), 40.7 (d, $J = 11.10$ Hz), 60.6, 62.8, 77.7 (d, $J = 15.9$ Hz), 94.6 (d, $J = 177.95$ Hz), 100.2 (d, $J = 2.83$ Hz), 173.8 (d, $J = 2.08$ Hz); δ_F (282 MHz, $CDCl_3$) -180.20 (d, $J = 49.5$ Hz), -181.03 (d, $J = 49.5$ Hz); HRMS (ESI) m/z 292.1921 $[M+NH_4]^+$ (Calcd. for $C_{14}H_{23}FO_4+NH_4^+$ 292.1924); IR (neat film): $\nu_{max}(cm^{-1})$ 2939 (-OEt), 1731 (C=O).

General procedure for the synthesis of (1*R, 3*R**, 4*R**)-ethyl-3-fluoro-4-hydroxycyclohexanecarboxylate (**2.50**)**



A catalytic amount of p-Toluene sulfonic acid was added to a racemic mixture of (1*R**, 3*R**, 4*R**)-ethyl-3-fluoro-4-(tetrahydro-2H-pyran-2-yloxy)cyclohexanecarboxylate **2.56** (1.7 g, 6.2 mmol) in methanol (8 mL) and was left to stir for 2 hours.⁸⁷ NaHCO₃ saturated solution was added (10 mL) to neutralise the acid. The methanol was evaporated *in vacuo*, and the solution was extracted with ethyl acetate (3×20 mL). The organic extracts were combined and washed with NaHCO₃ saturated solution (30 mL) and NaCl saturated solution (30 mL), dried over MgSO₄, filtered, evaporated *in vacuo*. The product was purified by flash chromatography on silica eluting with a mixture of dichloromethane and ethyl acetate (90: 10) to give the *title compound* **2.50** as a colourless oil (1.09 g, 5.74 mmol, 93%).

(1*S**, 3*R**, 4*R**)-ethyl-3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** (524 mg, 2.76 mmol) in THF (5 mL) was slowly added over a period of 10 minutes to a solution of sodium hydride (138mg, 5.75 mmol) in THF (55 mL) at –20°C. The reaction was left to stir at for 18 hours. Phtalamide (1g) was then added and left to stir for 1 hour at –20°C, then *tert*-butanol (4 mL) was added and left to stir for another hour at –20°C. The solution was left to heat up to room temperature. Then ether was added (100 mL), and the solution was extracted. The organic extract was washed with NaHCO₃ saturated solution and NaCl saturated solution, then dried over MgSO₄, filtered, evaporated *in vacuo* and purified by 2 successive flash chromatography on silica eluting with a mixture of petroleum ether and ethyl acetate (90/10) and then (85/15), to give the *title compound* **2.50** as a light orange oil (123 mg, 0.64 mmol, 23%) and recovered starting material (330 mg, 1.74 mmol, 63%).

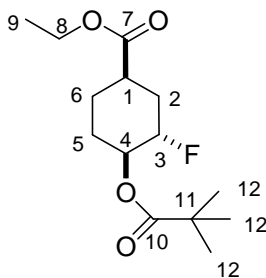
Potassium *tert*-butoxide (2.13 g, 18.9 mmol) was added to (1*S**, 3*R**, 4*R**)-ethyl-3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** (3 g, 15.79 mmol) in freshly distilled toluene under nitrogen. The reaction vessel was heated under reflux (in an oil bath pre-heated to 120°C) for 30 minutes. The reaction vessel was allowed to cool to room temperature, whilst stirring exposed to the atmosphere. After 10 minutes the reaction mixture was filtered through a cotton wool plug and washed with ethyl acetate (100 mL). The solvent was removed *in vacuo*, ensuring that the water bath temperature did

not exceed 30°C to avoid loss of the volatile product (2.8g crude). The compound was purified by flash chromatography on silica eluting with a gradient mixture of petroleum ether and ethyl acetate (9:1) to (85:15) petroleum ether /ethyl acetate, to give the *title compound* as a light orange oil (1.01 g, 5.3 mmol, 34%).

Potassium *tert*-butoxide (532 mg, 4.74 mmol) was added to a 4 : 1 mixture of (1*S**, 3*R**, 4*R**)-ethyl-3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** and (1*R**, 3*R**, 4*R**)-ethyl-4-fluoro-3-hydroxycyclohexanecarboxylate **2.47** (750 mg, 3.95 mmol) in freshly distilled toluene under nitrogen. The reaction vessel was then heated under reflux (in an oil bath pre-heated to 120°C) for 30 minutes. The reaction vessel was then allowed to cool to room temperature, whilst stirring exposed to the atmosphere. After 10 minutes the reaction mixture was filtered through a cotton wool plug and washed with ethyl acetate (100 mL). The solvent was removed *in vacuo*, ensuring that the water bath temperature did not exceed 30°C to avoid loss of the volatile product (2.8g crude). The compound was purified by flash chromatography on silica eluting with a gradient mixture of petroleum ether and ethyl acetate (9:1) to (85:15) petroleum ether /ethyl acetate, to give the *title compound* **2.50** as a light orange oil (178 mg, 0.937 mmol, 24%).

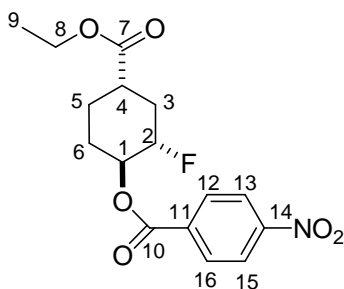
δ_{H} (400MHz, CDCl_3) 1.25 (t, $J = 7.1$ Hz, 3H, H9), 1.35-1.50 (m, 2H, H5, H6), 1.63-1.70 (m, 1H, H2), 1.96-2.02 (m, 1H, H6), 2.06-2.12 (m, 1H, H5), 2.33-2.43 (m, 2H, H1, H2), 3.61-3.73 (m, 1H, H4), 4.13 (q, $J = 7.1$ Hz, 2H, H8), 4.35 (dddd, $J = 50.7, 11.2, 8.6, 4.7$ Hz, 1H, H3); δ_{C} (75 MHz, CDCl_3) 14.0 (C9), 26.4 (C6), 30.2 (d, $J = 7.6$ Hz, 1C, C5), 32.4 (d, $J = 19.9$ Hz, 1C, C2), 40.9 (d, $J = 11.1$ Hz, 1C, C1), 60.7 (C8), 72.7 (d, $J = 18.0$ Hz, C4), 95.5 (d, $J = 175.3$ Hz, C3), 173.9 (C7); IR 1731.4 (C=O), 3438.8 (-OH), 2941.4 (-CH); δ_{F} (282 MHz, CDCl_3) -186.2; HRMS (ESI) m/z 191.1080 $[\text{M}+\text{H}]^+$ (Calcd. for $\text{C}_9\text{H}_{15}\text{FO}_3+\text{H}^+$ 191.1083).

General procedure for the synthesis of (1*R, 3*S**, 4*S**)-ethyl 3-fluoro-4-(pivaloyloxy)cyclohexanecarboxylate (**2.55**)**



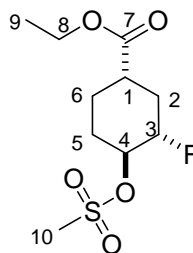
Pivalic acid (92 mg, 0.90 mmol) was added to thionyl chloride (106 mg, 0.89 mmol) in DCM (2 mL) and refluxed for 2 hours under nitrogen. The solution was left to cool down, then (1*R**, 3*S**, 4*S**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** (113 mg, 0.59 mmol) in DCM (1 mL) was added and left to stir over night under nitrogen. DCM (10 mL) was added, the organic solution was washed with saturated NaHCO₃ solution (3×5 mL), dried over Na₂SO₄, filtered, evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petrol and ethyl acetate (95:5) to give the *title compound* **2.55** as yellow crystals (47 mg, 0.17 mmol, 29%): δ_{H} (300MHz, CDCl₃) 1.23 (s, 9H, H12), 1.27 (t, $J = 7.1$ Hz, 3H, H9), 1.67-2.13 (m, 6H, H 2,6,5), 2.67-2.77 (m, 1H, H1), 4.15 (q, $J = 7.1$ Hz, 2H, H8), 4.71 (dddd, $J = 47.0, 4.6, 4.3, 4.3$ Hz, 1H, H3), 4.91-5.00 (m, 1H, H4); δ_{C} (75 MHz, CDCl₃) 14.2 (C9), 23.3 (C6), 25.0 (C5), 27.1 (3C, C12), 29.4 (d, $J = 18$ Hz, C2), 37.2 (d, $J = 3.8$ Hz, C1), 38.8 (C11), 60.6 (C8), 68.8 (d, $J = 27.0$ Hz, C4), 87.6 (d, $J = 172.5$ Hz, C3), 174.6 (C10), 177.3 (C7); δ_{F} (282 MHz, CDCl₃) -187.9; HRMS (CI) m/z 275.1658 [M+H]⁺ (Calcd. 275.1659 For C₁₄H₂₃FO₄+H⁺); IR (neat film): ν_{max} (cm⁻¹) 2917.2 (-OEt), 2849.0 (-OR), 1719.4 (C=O)

General procedure for the synthesis of (1*S, 2*S**, 4*S**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl 4-nitrobenzoate (**2.57**)⁸⁵**



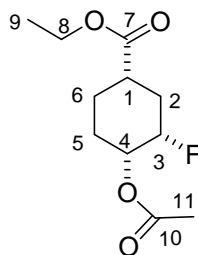
4-Nitro-benzoyl chloride (205 mg, 1.11 mmol) was added to 3-fluoro-4-hydroxycyclohexanecarboxylic acid ethyl ester **2.50** (106 mg, 0.56 mmol) in pyridine (3 mL) under nitrogen and was left to stir over night. Water (6 mL) was added, and the solution was extracted with ethyl acetate (3×20 mL). The organic extracts were combined and successively washed with oxalic acid solution (10%, 20 mL), cold NaHCO₃ solution (10%, 20 mL), and water (2× 20 mL) and dried over Na₂SO₄, filtered, evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petrol and ethyl acetate (95:5) to give the *title compound* **2.57** as white crystals (145 mg, 0.43 mmol, 77%): δ_{H} (400MHz, CDCl₃) 1.28 (t, J = 7.1 Hz, 3H, H9), 1.47-1.69 (m, 2H, H5, H6), 1.79-1.91 (m, 1H, H3), 2.06-2.11 (m, 1H, H5), 2.27-2.35 (m, 1H, H6), 2.42-2.56 (m, 2H, H4, H3), 4.17 (q, J = 7.1 Hz, 2H, H8), 4.65 (dddd, J = 50.4, 11.4, 8.9, 4.9 Hz, 1H, H2), 5.14 (ddt, J = 10.8, 9.0, 5.0 Hz, 1H, H1), 8.22 (d, J = 8.7 Hz, 2H, H13, H15), 8.29 (d, J = 8.7 Hz, 2H, H12, H16); δ_{C} (75 MHz, CDCl₃) 14.0 (C9), 26.1 (C5), 28.2 (d, J = 6.6 Hz, C6), 32.8 (d, J = 19.9 Hz, C3), 40.4 (d, J = 10.6 Hz, C4), 60.9 (C8), 75.5 (d, J = 17.8 Hz, C1), 91.2 (d, J = 181.6 Hz, C2), 123.6 (s, 2C, C13, C15), 130.9 (s, 2C, C12, C16), 135.5 (C11), 150.7 (s, 1C), 164.1 (C10), 173.3 (C7); δ_{F} (282 MHz, CDCl₃) -181.9 (d, J = 50.8 Hz) HRMS (ESI) m/z 357.1452 [M+NH₄]⁺ (Calcd. 357.1456 for C₁₆H₁₈FO₆+NH₄⁺), X-ray structure available.

General procedure for the synthesis of (1*S, 3*S**, 4*S**)-ethyl 3-fluoro-4-(methylsulfonyloxy)cyclohexanecarboxylate (**2.60**)**⁸⁹



DMAP (349 mg, 2.82 mmol) was added to a solution of (1*R**, 3*R**, 4*R**)-ethyl-3-fluoro-4-hydroxycyclohexanecarboxylate **2.50** (261 mg, 1.37 mmol) and *N*-Ethyl-diisopropylamine (530.4 mg, 4.1 mmol) in DCM (25 mL) at -10°C stirring under nitrogen and was left to stir for 30 minutes. Mesyl chloride (325 mg, 2.84 mmol) was added and left to stir for a further 2 hours at -10°C stirring under nitrogen to completion. Iced water (10 mL) was added and the organic phase was extracted. The aqueous phase was extracted with diethyl ether (3×10 mL). The DCM and diethyl ether extracts were combined, washed with NaCl saturated aqueous solution (2×10 mL), dried over MgSO₄, filtered, and evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petroleum ether and ethyl acetate (9 : 1), to give the *title compound* **2.60** as a white solid (170 mg, 0.634 mmol, 60%): δ_{H} (400 MHz, CDCl₃) 1.25 (t, J = 7.1 Hz, 3H, H9), 1.49-1.82 (m, 3H, H2, H5, H6), 2.02-2.11 (m, 1H, H6), 2.25-2.48 (m, 3H, H1, H2, H5), 3.06 (m, 3H, H10), 4.09 (q, J = 7.1 Hz, 2H, H8), 4.44-4.63 (m, 2H, H3, H4), δ_{C} (75 MHz, CDCl₃) 173.0 (C7), 91.2 (d, J = 180.5 Hz, C3), 82.0 (d, J = 17.1 Hz, C4), 60.8 (C8), 40.0 (d, J = 10.3 Hz, C1), 38.1 (C10), 32.7 (d, J = 20.0 Hz, C2), 29.9 (d, J = 1.7 Hz), 29.8 (d, J = 6.3 Hz, C5), 13.9 (C9); δ_{C} (282 MHz, CDCl₃) -179.6 (d, J = 50.8 Hz); HRMS (ESI) m/z 286.1120 [M+NH₄]⁺ (Calcd. for C₁₀H₁₇FO₅S +NH₄⁺ 286.1119); IR (neat film): ν_{max} (cm⁻¹) 2933 (-OEt), 1721 (C=O).

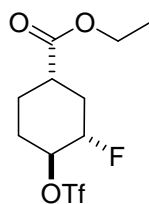
General procedure for the synthesis of (1*S, 3*S**, 4*R**)-ethyl 4-acetoxy-3-fluorocyclohexanecarboxylate (**2.61**)⁹²**



(1*S**, 3*S**, 4*S**)-ethyl 3-fluoro-4-(methylsulfonyloxy)cyclohexanecarboxylate **2.60** (30 mg, 0.11 mmol) was added to a solution of sodium acetate (185 mg, 2.8 mmol) in DMF water 80: 20 mixture (5 mL) was refluxed under nitrogen for 2 days. Ethyl acetate (10 mL) was added. The mixture was washed with water (3×5 mL). The organic phase was dried over MgSO₄, filtered, and evaporated *in vacuo* and purified by flash chromatography on silica eluting with a mixture of petroleum ether and DCM (1 : 1), to give the *title compound* **2.61** as (3 mg, 12.9 μmol, 12%)

δ_{H} (400MHz, CD₃OD) 1.28 (t, J = 7.1 Hz, 3H, H₉), 1.32 (s, 3H), 1.54-1.68 (m, 2H), 1.76-1.84 (m, 1H), 1.91-2.04 (m, 2H), 2.11-2.21 (m, 1H), 2.43-2.51 (m, 1H, H₁), 3.99-4.05 (m, 1H, H₄), 4.16 (q, J = 7.1 Hz, 2H, H₈), 4.56 (dddd, J = 47.2, 10.4, 4.2, 2.7 Hz, 1H, H₃); δ_{C} (75 MHz, CD₃OD) 15.3 (C₉), 23.9 (C₁₁), 30.6 (d, J = 21.2 Hz, C₂), 30.6 (d, J = 6.7 Hz, C₅), 31.6 (C₆), 42.0 (d, J = 9.5 Hz, C₁), 62.5 (C₈), 69.1 (d, J = 17.4 Hz, C₄), 94.2 (d, J = 176.8 Hz, C₃), 177.1 (C₁₀), 177.1 (C₇); MS (CI) m/z 250.2 [M+NH₄]⁺ (Calcd. for C₁₀H₁₇FO₅S +NH₄⁺ 250.2)

General procedure for the synthesis of (1*S, 3*S**, 4*S**)-ethyl-3-fluoro-4-(trifluoromethylsulfonyloxy) cyclohexanecarboxylate (**2.62**)**

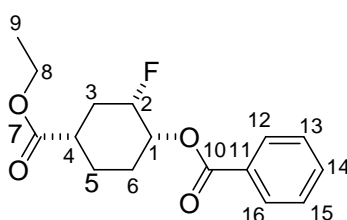


Pyridine (0.1 mL, 1.23 mmol) and a racemic mixture of (1*R**, 3*R**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.50** (120 mg, 0.632 mmol) in dry dichloromethane (1 mL) was added drop wise over a period of 10 minutes to a solution of trifluoromethanesulfonic anhydride (355 mg, 1.26 mmol) in dry dichloromethane (1 mL) previously stirred for 10 minutes at 0 °C under nitrogen. The reaction mixture was stirred for an extra 30 minutes at 0 °C, then stirred at room temperature. The reaction went to completion after 2 hours and dichloromethane (10 mL) was added. The organic

solution was washed with ice cold water (3× 5 mL), dried over MgSO₄, filtered and the solvent removed *in vacuo*, to give the crude *title compound* **2.62** as a colourless oil (137 mg, 67%).

δ_{H} (400 MHz, CDCl₃) 1.26 (t, J = 7.2 Hz, 3), 1.70-1.82 (m, 2H), 2.06-2.03 (m, 1H), 2.10-2.15 (m, 1H), 2.33-2.42 (m, 2H), 2.51-2.55 (m, 1H), 4.14 (q, J = 7.1 Hz, 2H), 4.50-4.63 (m, 1H), 4.80-4.83 (m, 1H); HRMS (EI) m/z 322.0495 [M]⁺ (Calcd. for C₁₀H₁₄F₄O₅S 322.0498).

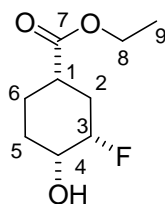
General procedure for the synthesis of (1*R, 2*S**, 4*S**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl benzoate (2.63)**



Tetrabutylammonium benzoate (666 mg, 1.8 mmol) was added to a racemic mixture of (1*S**, 3*S**, 4*S**)-ethyl-3-fluoro-4-(trifluoromethylsulfonyloxy) cyclohexanecarboxylate **2.62** (131 mg, 0.41 mmol) in freshly distilled toluene (18 mL) and stirred under nitrogen over night. The solvent was removed *in vacuo*. The product was purified by 2 successive flash chromatography: the first one on silica eluting with a mixture of hexane and ethyl acetate (95: 5), the second one on silica eluting with dichloromethane, to give the *title compound* **2.63** as white solid crystals (75 mg, 61%).

δ_{H} (300 MHz, CDCl₃) 1.26 (t, J = 7.1 Hz, 3H, H₉), 1.60-1.66 (m, 1H, H₆), 1.75-1.92 (m, 2H, H₅, H₆), 2.17-2.32 (m, 3H, H₃, H₃, H₅), 2.48 (dddd, J = 9.4, 9.4, 4.7, Hz, 1H, H₄), 4.16 (q, J = 7.1 Hz, 2H, H₈), 4.71 (dddd, J = 46.5, 10.1, 4.5, 2.8 Hz, 1H, H₂), 5.46 (dddd, J = 11.0, 5.3, 2.6, 2.6 Hz, 1H, H₁), 7.44 (dd, J = 7.5, 7.5 Hz, 2H, H₁₃, H₁₅), 7.56 (tt, J = 7.4, 1.4 Hz, 1H, H₁₄), 8.06 (dd, J = 8.4, 1.4 Hz, 2H, H₁₂, H₁₆); δ_{C} (75 MHz, CDCl₃) 14.0 (C₉), 22.4 (C₆), 26.3 (C₅), 29.6 (d, J = 21.2 Hz, 1C, C₃), 39.5 (d, J = 9.2 Hz, 1C, C₄), 60.6 (C₈), 69.7 (d, J = 16.8 Hz, 1C, C₁), 89.6 (d, J = 182.8 Hz, 1C, C₁), 128.4 (C₁₃, C₁₅), 129.7 (C₁₂, C₁₆), 130.1 (C₁₁), 133.2 (C₁₄), 165.8 (C₁₀), 173.8 (C₇); δ_{F} (282 MHz, CDCl₃) -185.7; HRMS (ESI) m/z 312.1606 [M+NH₄]⁺ (Calcd. for C₁₆H₁₉FO₄+NH₄⁺ 312.1611); IR (neat film): ν_{max} (cm⁻¹) 2964.5 (-OEt), 1728.7 (C=O), 1602.0, 1584.0, 865.2 (aromatic).

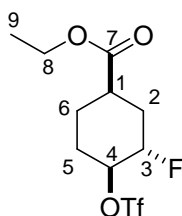
General procedure for the synthesis of (1*R, 3*R**, 4*S**)-ethyl-3-fluoro-4-hydroxycyclohexanecarboxylate (**2.58**)**



A catalytic amount of sodium was stirred in dry ethanol (2 mL) under nitrogen to form sodium ethoxide *in situ*. A racemic mixture of (1*R**, 2*S**, 4*S**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl benzoate **2.63** (75 mg, 0.25 mmol) in dry ethanol (2 mL) was added to the previous sodium ethoxide solution and stirred under nitrogen over night. pH 7 phosphate buffer was added (20 mL) until pH was close to neutral, then the ethanol was removed *in vacuo*. The aqueous solution was extracted with ethyl acetate (3× 20 mL). The organic extracts were combined and washed with saturated NaHCO₃ (30 mL), saturated aqueous NaCl solution (30 mL), dried on MgSO₄, and filtered. The solvent was removed *in vacuo* and purified by flash chromatography on silica eluting with a mixture of hexane and ethyl acetate (9:1) to give the *title compound* **2.58** as a yellow oil (42 mg, 0.22 mmol, 88%) and compound **2.59** as a yellow oil (2 mg, 0.01 mmol, 4%).

δ_{H} (300 MHz, CDCl₃) 1.25 (t, J = 7.1 Hz, 3H, CH₂-CH₃), 1.40-1.53 (m, 1H, H5), 1.69-1.70 (m, 1H, H6), 1.77-1.90 (m, 1H, H6), 1.97-2.25 (m, 3H, H2, H2, H5), 2.29-2.38 (m, 1H, H1), 4.06-4.11 (m(o), 1H, H4), 4.14 (q, J = 7.1 Hz, 2H, H8), 4.55 (dddd, J = 47.0, 10.5, 4.8, 2.8 Hz, 1H, H3); δ_{C} (75 MHz, CDCl₃) 14.0 (CH₂-CH₃), 21.6 (C6), 28.2 (d, J = 20.9 Hz, C2), 29.6 (C5), 40.2 (d, J = 10.3 Hz, C1), 60.6 (C8), 66.9 (d, J = 17.9 Hz, C4), 92.5 (d, J = 175.0 Hz, C3), 174.0 (C7); δ_{F} (282 MHz, CDCl₃) -185.3; HRMS (ESI) m/z 191.1078 [M+H]⁺ (Calcd. for C₉H₁₅FO₃+H⁺ 191.1083).

General procedure for the synthesis of (1*R, 3*S**, 4*S**)-ethyl-3-fluoro-4-(trifluoromethylsulfonyloxy)cyclohexanecarboxylate (**2.64**)**

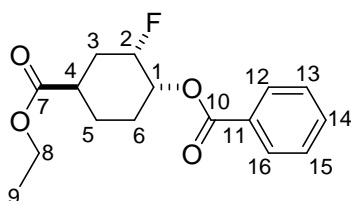


Pyridine (0.07 mL, 0.86 mmol) and a racemic mixture of (1*S**, 3*R**, 4*R**)-ethyl 3-fluoro-4-hydroxycyclohexanecarboxylate **2.44** (87 mg, 0.46 mmol) in dry dichloromethane (1 mL) was added drop wise over a period of 10 minutes to a solution of trifluoromethanesulfonic anhydride (242.3 mg, 0.86 mmol) in dry dichloromethane

(1 mL) previously stirred for 10 minutes at 0 °C under nitrogen. The reaction mixture was stirred for 30 minutes at 0 °C and then stirred at room temperature. The reaction went to completion after 2 hours as evident by TLC and dichloromethane (10 mL) was added and quenched with adding ice cold water. The organic solution was washed with ice cold water (3× 5 mL), dried over MgSO₄, filtered and the solvent removed *in vacuo*, to give the crude *title compound* **2.64** as a colourless oil (113 mg, 76%).

δ_{H} (400 MHz, CDCl₃) 1.27 (t, J = 7.14 Hz, 3H), 1.76-1.83 (m, 1H), 1.93-2.06 (m, 3H), 2.10-2.17 (m, 1H), 2.26-2.41 (m, 1H), 2.75-2.80 (m, 1H), 4.17 (q, J = 7.1 Hz, 2H), 4.78-4.94 (m, 2H); HRMS (EI) m/z 322.0493 [M]⁺ (Calcd. for C₁₀H₁₄F₄O₅S 322.0498).

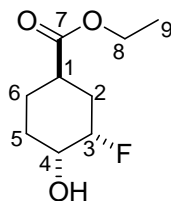
General procedure for the synthesis of (1*R, 2*S**, 4*R**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl benzoate (2.65)**



Tetrabutylammonium benzoate (290 mg, 0.80 mmol) was added to a racemic mixture of (1*R**, 3*S**, 4*S**)-ethyl-3-fluoro-4-(trifluoromethylsulfonyloxy) cyclohexanecarboxylate **2.64** (110 mg, 0.34 mmol) in freshly distilled toluene (15 mL) and stirred under nitrogen over night. The solvent was removed *in vacuo*. The product was purified by 2 successive flash chromatography: the first one on silica eluting with a mixture of hexane and ethyl acetate (95: 5), the second one on silica eluting with dichloromethane, to give the *title compound* **2.65** as white crystals (64 mg, 65%).

δ_{H} (300 MHz, CDCl₃) 1.27 (t, J = 7.1 Hz, 1H, H₉), 1.61-1.91 (m, 2H, H₃, H₅), 1.95-2.07 (m, 2H, H₆, H₅), 2.09-2.20 (m, 1H, H₆), 2.36-2.45 (m, 1H, H₃), 2.76 (tt, J = 12.1, 3.9 Hz, 1H, H₄), 4.15 (q, J = 7.1 Hz, 2H, H₈), 4.95-5.13 (m, 2H, H₂, H₁), 7.45 (t, J = 7.6 Hz, 2H, H₁₃, H₁₅), 7.57 (t, J = 7.4 Hz, 1H, H₁₄), 8.07 (dd, J = 8.3, 1.1 Hz, 2H, H₁₂, H₁₆); δ_{C} (75 MHz, CDCl₃) 14.0 (C₉), 24.7 (d, J = 3.0 Hz, 1C, C₅), 26.2 (C₆), 31.9 (d, J = 20.3 Hz, 1C, C₃), 36.5 (d, J = 2.0 Hz, 1C, C₄), 60.6 (C₈), 72.5 (d, J = 17.8 Hz, 1C, C₁), 88.2 (d, J = 177.3 Hz, 1C, C₂), 128.4 (C₁₃, C₁₅), 129.8 (C₁₂, C₁₆), 130.0 (C₁₁), 133.2 (C₁₄), 166.0 (C₁₀), 174.7 (C₇); δ_{F} (282 MHz, CDCl₃) -200.6; HRMS (ESI) m/z 312.1608 [M+NH₄]⁺ (Calcd. for C₁₆H₁₉FO₄+NH₄⁺ 312.1611); IR (neat film): ν_{max} (cm⁻¹) 2956.0 (-OEt), 1729.9 (C=O), 1601.9, 1584.5, 859.1 (aromatic).

General procedure for the synthesis of (1*S**, 3*R**, 4*S**)-ethyl-3-fluoro-4-hydroxycyclohexanecarboxylate (**2.59**)



A catalytic amount of sodium was stirred in dry ethanol (2 mL) under nitrogen to form sodium ethoxide *in situ*. A racemic mixture of (1*R**, 2*S**, 4*R**)-4-(ethoxycarbonyl)-2-fluorocyclohexyl benzoate **2.65** (52 mg, 0.176 mmol) in dry ethanol (2 mL) was added to the previous sodium ethoxide solution and stirred under nitrogen over night. pH 7 phosphate buffer was added (15 mL) until pH was close to neutral, then the ethanol was removed *in vacuo*. The aqueous solution was extracted with ethyl acetate (3× 20 mL). The organic extracts were combined and washed with saturated NaHCO₃ (30 mL), saturated aqueous NaCl solution (30 mL), dried on MgSO₄, and filtered. The solvent was removed *in vacuo* and purified by flash chromatography on silica eluting with a mixture of hexane and ethyl acetate (9:1) to give the *title compound* **2.59** as transparent oil (19 mg, 57%) and the epimerised product **2.58** (11 mg, 33%).

δ_{H} (300 MHz, CDCl₃) 1.25 (t, J = 7.14 Hz, 3H, H₉), 1.55-1.70 (m, 2H, H₂, H₆), 1.73-2.06 (m, 4H, H₅, H₅, H₆, -OH), 2.27-2.37 (m, 1H, H₂), 2.62 (tt, J = 11.5, 11.5, 3.9, 3.9 Hz, 1H, H₁), 3.62 (dddd, J = 26.4, 10.9, 4.7, 2.3 Hz, 1H, H₄), 4.12 (q, J = 7.14 Hz, 2H, H₈), 4.86 (dddd, J = 50.23, 4.45, 2.15, 2.15 Hz, 1H, H₃); δ_{C} (75 MHz, CDCl₃) 14.0 (C₉), 26.0 (C₆), 28.3 (d, J = 3.25 Hz, 1C, C₅), 31.5 (d, J = 20.43 Hz, 1C, C₂), 36.7 (d, J = 2.27 Hz, 1C, C₁), 60.5 (s, 1C, C₈), 70.0 (d, J = 19.4 Hz, 1C, C₄), 91.1 (d, J = 171.9 Hz, 1C, C₃), 175.1 (C₇); δ_{F} (282 MHz, CDCl₃) -203.1; HRMS (ESI) m/z 208.1340 [M+NH₄]⁺ (Calcd. for C₉H₁₅FO₃+NH₄⁺ 208.1343).

3 ADMINISTERING THE SHIKIMIC ACID ANALOGUES TO S. HYGROSCOPICUS MG-210 CULTURES

General Procedures

Microbial culturing was carried out under sterile conditions in a laminar flow cabinet (Nuair, NU-440). A New Brunswick orbital incubator was used for the culturing. The pH was adjusted using a pH meter (Mettler Toledo – MP-220)

Construction of rapQONMLKJI Deficient *S. hygroscopicus* NRRL 5491

S. hygroscopicus MG2-10 deficient in shikimic acid biosynthesis was constructed by double recombination as detailed below:

Plasmid pMG144 contains homology to terminal regions of the rapQONMLKJI gene sequence which, after double recombination, results in disruption of rapQONMLKJI. It is based on the suicide vector pMG55 harbouring rpsL to confer dominant sensitivity to streptomycin as a positive marker for the second crossover.

S. hygroscopicus NRRL 5491 was selected for streptomycin resistance to give *S. hygroscopicus* MG1C. *S. hygroscopicus* MG2-10 was constructed by double recombination of pMG144 with *S. hygroscopicus* MG1C.

WS spore stocks were prepared by Hrvoje Petkovic on 08.01.04 and a single vial was used for the following experiments (19/05/08).

General Procedures

Working stocks were prepared from oatmeal (ISP3) or MAM plates. Strains were inoculated to give confluent growth. Cultures were incubated for 2-3 weeks at 28°C. White aerial hyphae form initially followed by a downy grey spore covering. The vegetative mycelia were cream, no melanin formation was observed. As the plates age beyond 2-3 weeks, spores turn black and hygroscopic and are unsuitable for working stocks. Spores were harvested from a 30 mL agar plate in 7 mL 20% v/v glycerol by washing the plates and filtering aseptically through glass wool to remove vegetative debris. Working stocks were aliquoted in 0.5 mL lots and stored at -80°C.

General details on the HPLC used for sample analysis in order to quantify rapamycin

The HPLC system comprised an Agilent HP1100 equipped with a Hyperclone 3µm BDS C18 130A column 150mm x 4.6mm (Phenomenex) heated to 50 °C. The gradient elution was from 55% mobile phase B to 95% mobile phase B over 10 minutes followed by an isocratic hold at 95% mobile phase B for 2 minutes with a flow rate of 1 mL/min. Mobile phase A was 10% acetonitrile:90% water, containing 10 mM ammonium acetate and 0.1% trifluoroacetic acid, mobile phase B was 90% acetonitrile:10% water, containing 10 mM ammonium acetate and 0.1% trifluoroacetic acid. Rapamycin

analogues were identified by the presence of the characteristic rapamycin triene, centred on $\lambda = 278$ nm. Samples were quantified based on a rapamycin calibration curve, measuring peak area at $\lambda = 280$ nm. The HPLC system described above was coupled to a Bruker Daltonics Esquire3000 electrospray mass spectrometer. The gradient elution was from 50% mobile phase B to 100% mobile phase B over 10 minutes followed by an isocratic hold at 100% mobile phase B for 3 minutes with a flow rate of 1 mL/min. Mobile phase A was water containing 0.1% formic acid, mobile phase B was acetonitrile containing 0.1% formic acid.. Positive negative switching was used over a scan range of 500 to 1000 Dalton.

Preparation of HPLC and LC-MS Samples from

-the whole culture broth

Whole culture broth (0.9 mL) was added to methanol (0.9 mL) in a 2 mL eppendorf, and was shaken for 30 minutes. The sample was then centrifuged (10 minutes, 13000 rpm) and the supernatant (150 μ L) was stored for HPLC quantification. 50 μ L of each sample was injected into an HPLC with diode array detection described above.

- acetonitrile extracts

Acetonitrile extract (500 μ L) was aliquoted in a 2 mL eppendorf and was centrifuged (10 minutes at 13000 rpm). The supernatant was aliquoted (200 μ L) and was stored for HPLC quantification. 50 μ L of each sample was injected into the previously described HPLC.

- ethyl acetate extracts

Ethyl acetate (200 μ L) was aliquoted in a 2 mL eppendorf and the solvent was removed using a speedy vac, and methanol (1 mL) was added and was shaken for 5 minutes and then was centrifuged for 10 minutes at 13000 rpm. The supernatant was aliquoted (150 μ L) in an HPLC vial. 50 μ L of sample was injected into the previously described HPLC.

Media preparation:

Rap V7 Medium (55L)

Soy flour (toasted nutrisoy)(275 g), white dextrin (1925 g), corn steep solids (220 g), ammonium sulphate (110g), lactic acid aqueous solution (80% per volume, 88 mL), calcium carbonate (385 g) were dissolved in distilled water and prepared to 55 L. The

solution was pH adjusted to 7.5 with sodium hydroxide. The solution was then sterilised by autoclaving (121 °C, 20 minutes). Glucose (550 g) was then added under sterile conditions after sterilisation.

MD6 (55L)

Soy bean flour (toasted nutrisoy) (1650 g), corn starch (1650 g), white dextrin W80 (1045 g), whole yeast (165 g), corn steep solids (55 g), ammonium sulphate (550 g), calcium carbonate (550 g), monopotassium phosphate (KH_2PO_4 , 137.5 g), potassium phosphate dibasic (K_2HPO_4 , 137.5 g), sodium chloride (275 g), magnesium sulphate heptahydrate (0.1375 g), manganese chloride tetrahydrate (0.55 g), zinc sulphate heptahydrate (2.75 g), ferrous sulphate heptahydrate (6.6 g), MES ($\text{C}_6\text{H}_{13}\text{NO}_4\text{S}$, 1166 g) were dissolved in distilled water and prepared to 55 L. The solution was pH adjusted to 6 using sodium hydroxide. Alpha-amylase (22 mL) was then added under sterile conditions. The solution was then sterilised by autoclaving (121 °C, 20 minutes).

3.1 Small Scale Feeding of Shikimic Acid Analogues

Preparation of Starter Cultures

Under sterile conditions RapV7 media (400 mL) and sterilised 40% (w/v) aqueous glucose solution (10 mL) were mixed to homogeneity. *S. hygroscopicus* MG2-10 spores (concentration, 70 µl) were used to inoculate 7 mL portions RapV7-Glucose medium in 10 sterile 50 mL falcon tubes which were sealed with foam bungs and shaken at 300 rpm (2.5 cm throw) at 28°C for 48 hours.

Preparation of Main Cultures

Under sterile conditions MD6 media (400 mL), 40% (w/v) fructose aqueous solution (20 mL) and filter sterilised 14% (w/v) L-lysine aqueous solution (5.6 mL) were mixed to homogeneity. The *S. hygroscopicus* seed cultures (0.5 mL) were used to inoculate 33 out of 34 7.45 mL portions of the MD6-fructose-L-lysine medium in 50 mL falcon tubes. The uninoculated culture medium was used as negative control. The falcon tubes were sealed with foam bungs and were shaken at 300 rpm (2.5 cm throw) at 26°C for 24 hours.

Administration of Shikimic Acid Analogues

Compounds **1.94**, **1.89**, **2.44**, **2.50**, **2.59**, **2.58**, **2.47**, **2.49** were prepared to a concentration of 0.32 molL^{-1} in methanol and were individually fed in quadruplet (50

μL , 16 μmol) to previously prepared *S. hygroscopicus* MG2-10 cultures to reach a final concentration of 2 mmolL^{-1} , except for compound **2.59** which was fed in triplicate. The falcon tubes were sealed with foam bungs and were shaken at 300 rpm (2.5 cm throw) at 26°C for 5 days. A single sample of uninoculated media and two unfed *S. hygroscopicus* cultures were used as negative controls. LC-MS samples were prepared for analysis from the whole broth. The results of these analyses are reported in the table.

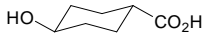
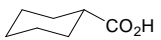
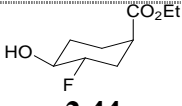
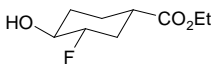
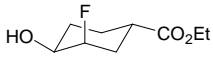
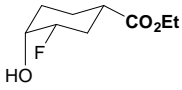

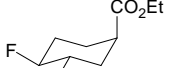
Starter acid analogue	Retention time	Peak area	Concentration mgL^{-1}	Average concentration	MS peaks detected
Uninoculated media	0	0	0	0.00	
Unfed culture	0	0	0		
Unfed culture	0	0	0		
 1.94	6.80	1694.1	28.24	28.57	$[\text{M}+\text{Na}]^+$
	6.80	1892.1	31.54		847.7
	6.80	1586.9	26.45		$[\text{M}+\text{formate}]^-$
	6.80	1682.4	28.05		870.5
 1.89	6.80	1579.3	26.33	27.43	$[\text{M}+\text{Na}]^+$
	6.80	1716.3	28.61		847.7
	6.79	1690.9	28.19		$[\text{M}+\text{formate}]^-$
	6.80	1594.9	26.59		870.5
 2.44	6.47	639.6	10.66	10.14	$[\text{M}+\text{Na}]^+$
	6.47	606.7	10.11		866.7
	6.47	569.6	9.50		$[\text{M}+\text{formate}]^-$
	6.46	617.3	10.29		888.4
 2.50	6.59	1716.1	28.61	27.96	$[\text{M}+\text{Na}]^+$
	6.59	1698.9	28.32		866.7
	6.59	1648.6	27.48		$[\text{M}+\text{formate}]^-$
	6.59	1645.1	27.43		888.6
 2.59	6.47	1464.2	24.41	26.41	$[\text{M}+\text{Na}]^+$
	6.47	1621.0	27.02		866.7
	6.47	1667.0	27.79		$[\text{M}+\text{formate}]^-$
 2.58	6.58	184.9	3.08	4.31	$[\text{M}+\text{Na}]^+$
	6.58	282.7	4.71		866.7
	6.58	262.7	4.38		
	6.58	304.1	5.07		
 2.47	6.88	253.1	4.22	4.68	$[\text{M}+\text{Na}]^+$
	6.88	283.0	4.72		866.7 and
	6.87	265.2	4.42		$[\text{M}+\text{formate}]^-$
	6.88	321.2	5.35		888.4
 2.49	6.58	421.9	7.03	7.08	$[\text{M}+\text{Na}]^+$
	6.58	462.7	7.71		866.7
	6.58	438.1	7.30		
	6.58	375.8	6.26		

Table 3.1: Incorporation results for the analytical feeding of the fluorohydrins.

3.2 Large scale feeding of the all equatorial fluorohydrin to *S. hygrosopicus* MG-210.

Under sterile conditions MD6 media (2 L), 40% (w/v) fructose aqueous solution (100 mL) and filter sterilised 14% (w/v) L lysine aqueous solution (28 mL) were mixed together to homogeneity. The *S. hygrosopicus* seed cultures (0.5 mL) were used to inoculate 300×7.45 mL portions of the MD6-fructose-L-lysine medium in 50 mL falcon tubes. The falcon tubes were sealed with foam bungs and were shaken at 300 rpm (2.5 cm throw) at 26°C for 24 hours.

Compound **2.50** was prepared in methanol to a concentration of 0.32 molL⁻¹ and was fed (50 µL, 16 µmol) to 300 previously prepared *S. hygrosopicus* MG2-10 cultures to reach a final concentration of 2 mmolL⁻¹. The cultures were sealed with foam bungs and were shaken at 300 rpm (2.5 cm throw) at 26°C for 5 days. Four analytical samples from the whole broth of four random falcon tubes were analysed by HPLC (**Table 3.2**). The 300 cultures were combined in a conical flask (1.5L). The tubes were washed with distilled water, and the washings were combined in a different flask (800 mL). The whole broth and the washings were analysed in triplicate by HPLC. The whole broth and the washings were combined after analysis. The cells were harvested by centrifugation (25 minutes at 3500 rpm) and the supernatant (1.8 L) was discarded after HPLC analysis in triplicate. Acetonitrile (2.75 L) was added to the cells, stirred for one hour and decanted. The cells were extracted a second time with acetonitrile (2.4 L) as previously. Both extracts and the aqueous phase were analysed in duplicate by HPLC. The second portion of ethyl acetate and the aqueous phase were discarded.

The acetonitrile extracts were combined, and the solvent removed *in vacuo*. Ethyl acetate (300 mL) and distilled water (400 mL) were added and stirred for 20 minutes and centrifuged (10 minutes, 3500 rpm). The organic phase was extracted and ethyl acetate (300 mL) was added and the procedure was repeated as before. Both extracts were analysed by HPLC in duplicate.

Ethyl acetate was removed *in vacuo*, yielding 2.246 g of crude material containing an estimated 31 mg of prerapamycin. The crude material was purified by flash chromatography on silica eluting with a mixture of ethyl acetate and hexane (6 : 4). All fractions were analysed by HPLC. Fractions with a high concentration of prerapamycin were combined (1.83 L), and fractions containing lower concentrations of prerapamycin were also combined separately (1.8 L). After HPLC analysis (**Table 3.2**) both fractions were combined and the solvent was removed *in vacuo*. The compound was dissolved in

acetonitrile (1.8 mL) and was injected in a preparative HPLC (Column – Waters Xterra MS C18, 10 micron, 250 mm x 19 mm diameter; flow rate 20 mL/min; solvent A = water, solvent B = acetonitrile; isocratic 45% B, RT of target compound = 21 mins). The solvent was removed *in vacuo* to reveal the target compound as an off-white amorphous solid (16.4 mg). The compound was analysed by NMR (¹H, ¹³C, DEPT, ¹⁹F, HMBC, HSQC, COSY, NOESY) and HRMS. The NMR analysis revealed a mixture of two compounds (**Table 3.3**).

Samples	mgL ⁻¹	Average concentration	Volume	Predicted mass	Total predicted mass
Falcon tube 1	23.26	21.30			
Falcon tube 2	20.18				
Falcon tube 3	20.17				
Falcon tube 4	21.52				
Whole broth	21.79 20.60 21.43	21.27		32 mg	35 mg
Washings	3.86 3.81 4.89	4.19	0.8 L	3 mg	
Supernatant	1.76 1.65 1.65	1.69	1.8 L	3mg discarded	
CH3CN EXT 1	10.25 10.21	10.23	2.75L	28mg	34mg
CH3CN EXT 2	2.55 2.56	2.56	2.4L	6mg	
EtOAc ext 1	102.03 108.61	105.3	0.3L	31.6mg	31.6 mg
EtOAc ext 2	0.00 0.00	0	0.3L	0	
Aqu ext	0.00 0.00	0	0.4L	0 DISCARD	
Column fractions with high concentration of compound	9.78 9.49 9.32	9.53	1.83L	17.4 mg	22.6 mg
Column fractions with low concentrations of compound	2.77 3.04 2.78	2.86	1.8L	5.2 mg	
2 preparative HPLC					16.4 mg

Table 3.2: Estimated and isolated quantities of fluoroprerapamycin 2.66 during the gramme scale feeding of compound 2.50

Feeding Experiment and fluoro-pre-rapamycin generation:

Fluoro-pre-rapamycin (2.66): HRMS (ESI) m/z 866.5234 ($M+Na$)⁺ (Calcd. for $C_{48}H_{74}NO_{10}F+Na^+$ 866.5194).

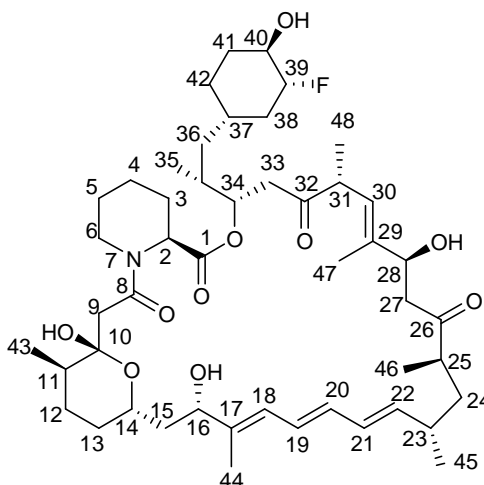


Table for NMR Chemical shifts of 2.66

δ_F (282 MHz, $CDCl_3$) -74.96, -75.85

Proton	δH		multiplicity		coupling		δC	
1	-	-					171.5	169.0
2	5.38	4.37					52.6	55.7
3a	1.78	1.53					26.2	26.7
3b	2.19	2.41						
4a	1.25	1.51					20.9	20.5
4b	1.75	1.71						
5a	1.49	1.29					25.1	24.4
5b	1.74	1.63						
6a	3.25	2.15					44.4	39.1
6b	3.84	4.47	brd	brd	13.1	13.4		
8							172.4	171.7
9a	2.57	2.41	d	d	13.8	16.1	40.2	38.8
9b	2.89	2.69	d	d	13.7	16.3		
10							98.6	98.4
10-OH		6.66		br. s				
11	1.49	1.54					38.6	38.6
12a	1.47	1.49					27.6	27.3
12b	1.62	1.69						
13a	1.34	1.26					31.9	32.3
13b	1.52	1.57						
14	3.89	4.22	dddd		11.4, 11.4, 2.0, 2.0		69.6	71.3

15a	1.53	1.46				40.6	40.6
15b	1.68	1.69					
16	4.08	4.22	dd		6.2, 6.2	75.6	76.0
17						138.4	141.6
18	6.23	6.09	d	d	12.2 10.5	125.1	122.6
19	6.33	6.39	dd	dd	14.6, 11.2 14.2, 11.2	127.7	128.6
20	6.19	6.00	dd	dd	14.7, 10.7 14.2, 10.8	132.2	131.2
21	6.05	6.06	dd	dd	14.9, 10.4 14.5, 10.6	130.5	130.2
22	5.31	5.19	dd	dd	14.9, 9.4 14.6, 9.9	139.0	139.4
23	2.16	2.12				37.3	39.8
24a	1.30	1.37		dd	13.2, 4.0	40.3	39.7
24b	1.69	1.98					
25	2.43	2.44				45.6	46.3
26						215.5	216.2
27a	2.54	2.45	dd	dd	16.7, 3.3 17.7, 2.1	46.4	48.0
27b	2.65	2.60	dd		16.7, 2.7		
28	4.34	4.40				72.0	72.1
29						139.6	139.7
30	5.37	5.44	d	d	9.8 9.8	123.7	125.4
31	3.25	3.39		qd	10, 6.8	46.4	45.6
32						208.8	209.0
33a	2.61	2.65	dd		18.3, 9.7	40.6	39.7
33b	2.63	2.95		ddd	18.6, 9.9, 2.5		
34	5.13	5.36	ddd		6.5, 6.5, 3.8	75.9	74.4
35	1.94	1.96				32.6	32.8
36a	1.09	1.05	dd		3.4, 3.4	37.5	37.5
36b	1.20	1.16	m	dd	7.5, 5.7		
37	1.41	1.45				32.9	33.0
38a	1.46	1.48				40.7	40.6
38b	1.68	1.66					
39	4.26 or 4.31- 4.21	4.37- 4.22	dddd		51.3, 10.2, 8.7, 5.0	96.2 (d, <i>J</i> = 173.8 Hz)	96.2(d, <i>J</i> = 173.8 Hz)
40	3.59	3.61				73.4	73.4
41a	1.28	1.29				30.9	30.9
41b	1.96	2.00					
42a	1.68	1.73				29.2	29.9
42b	0.84	0.90					
43	0.95	0.95	d	d	6.5 6.5	16.8	16.9
44	1.65	1.79	s	s		12.7	15.7
45	1.02	1.00	d	d	6.3 6.0	21.6	21.7
46	1.01	1.03	d	d	6.1 7.2	16.6	19.1
47	1.59	1.49	d	d	1.0 1.1	13.1	11.8
48	1.08	1.01	d	s	6.8 7.1	16.2	14.6
49	0.89	0.90	d	d	6.1 6.5	16.0	15.0

Table 3.3: ^1H and ^{13}C chemical shifts for both fluoroprerapamycins.

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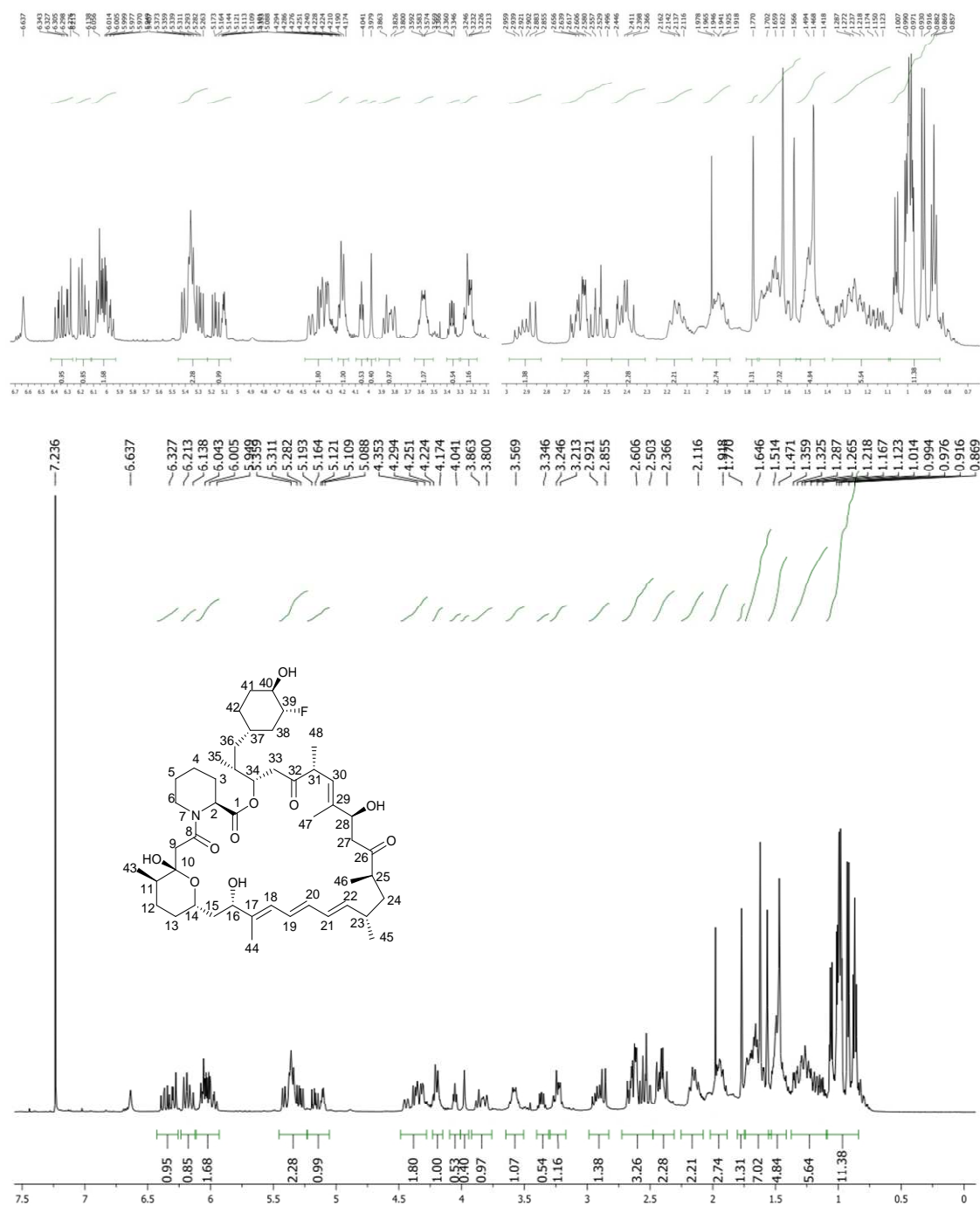
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APPENDIX 1: ^1H NMR Spectrum of Fluoroprerapamycin

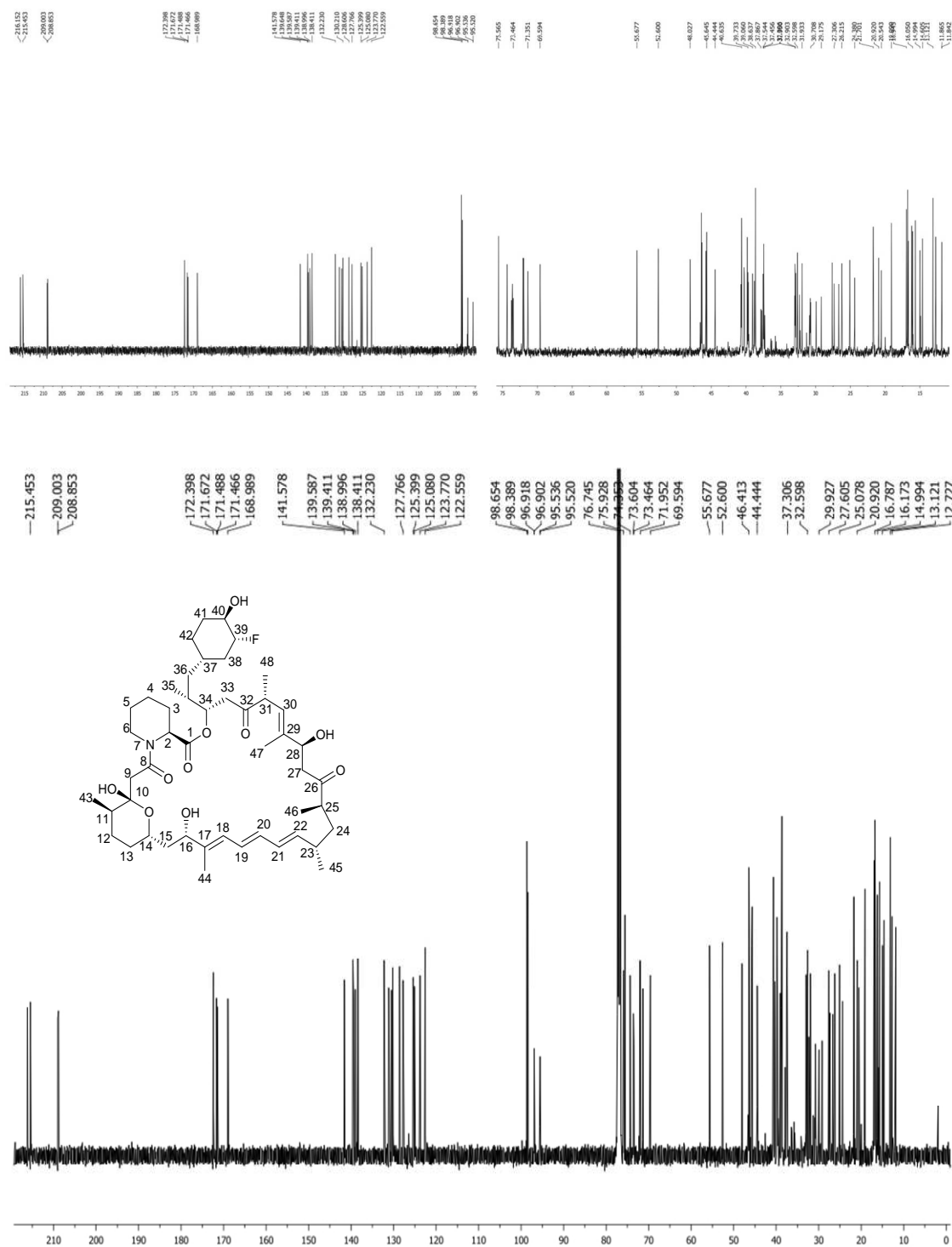
500 MHz, CDCl_3



^1H NMR spectrum of fluoro-pre-rapamycin (2.66).

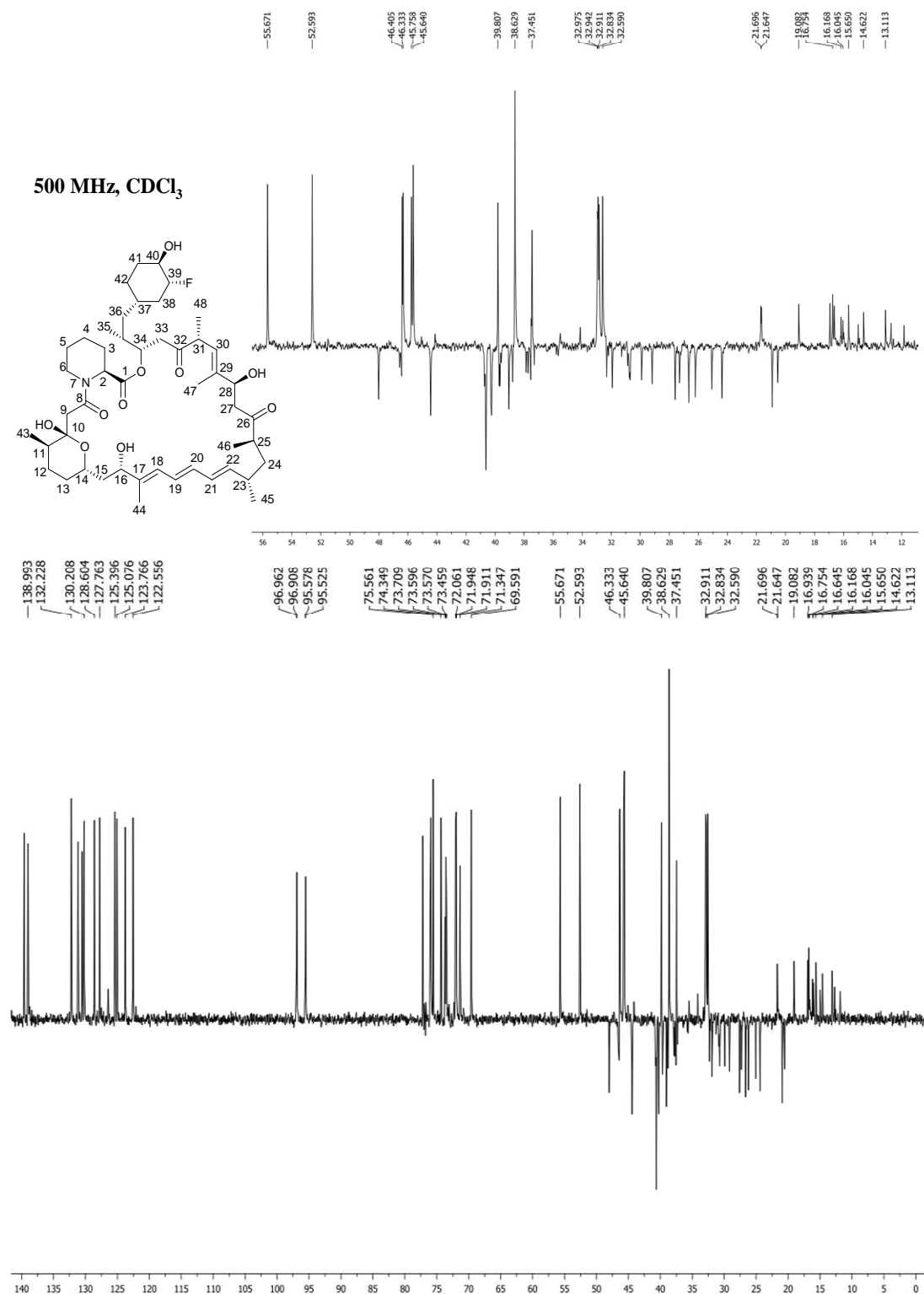
APPENDIX 2: ^{13}C NMR Spectrum of Fluoroprerapamycin

500 MHz, CDCl_3



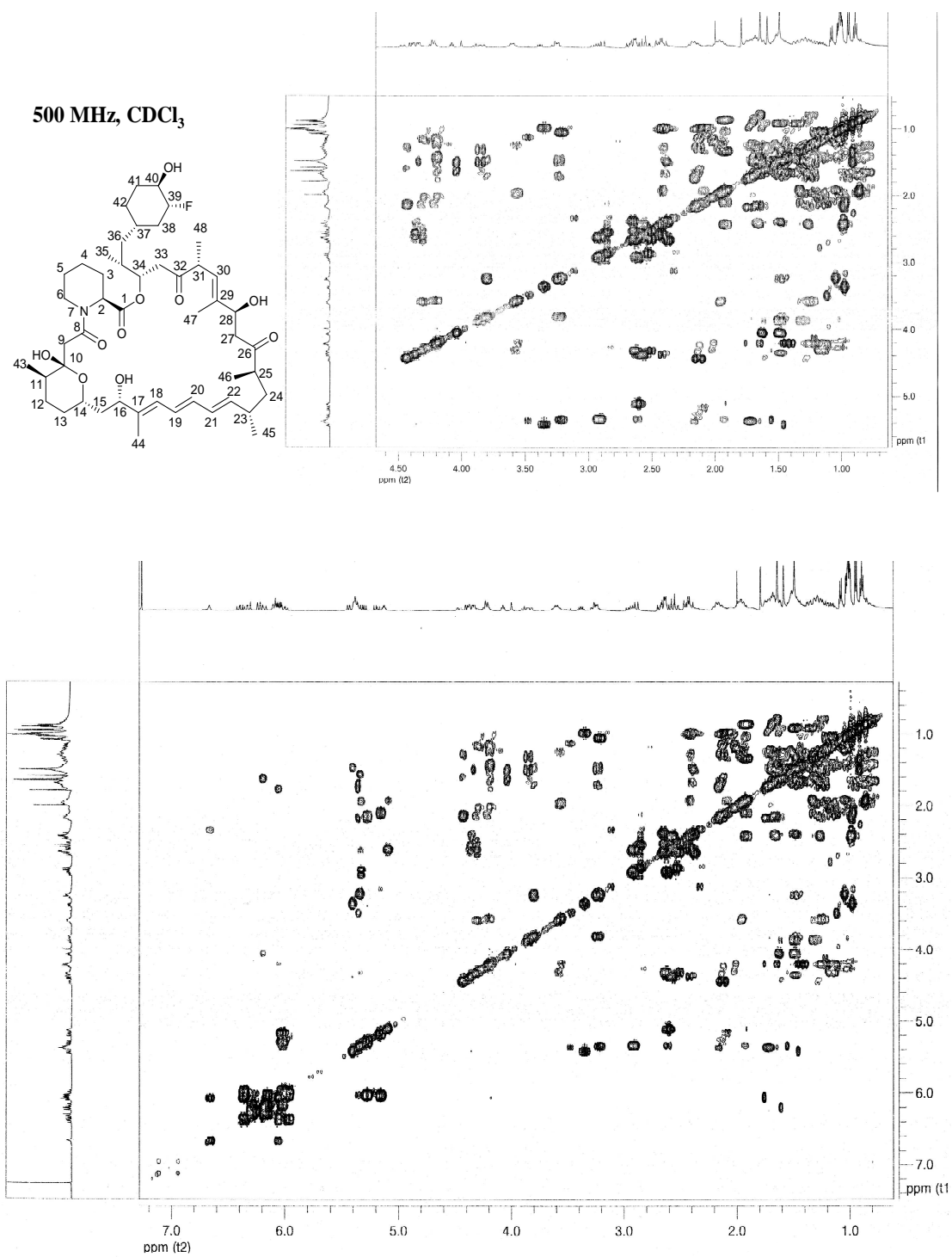
^{13}C NMR spectrum of fluoro-pre-rapamycin (2.66).

APPENDIX 3: DEPT NMR Spectrum of Fluoroprerapamycin



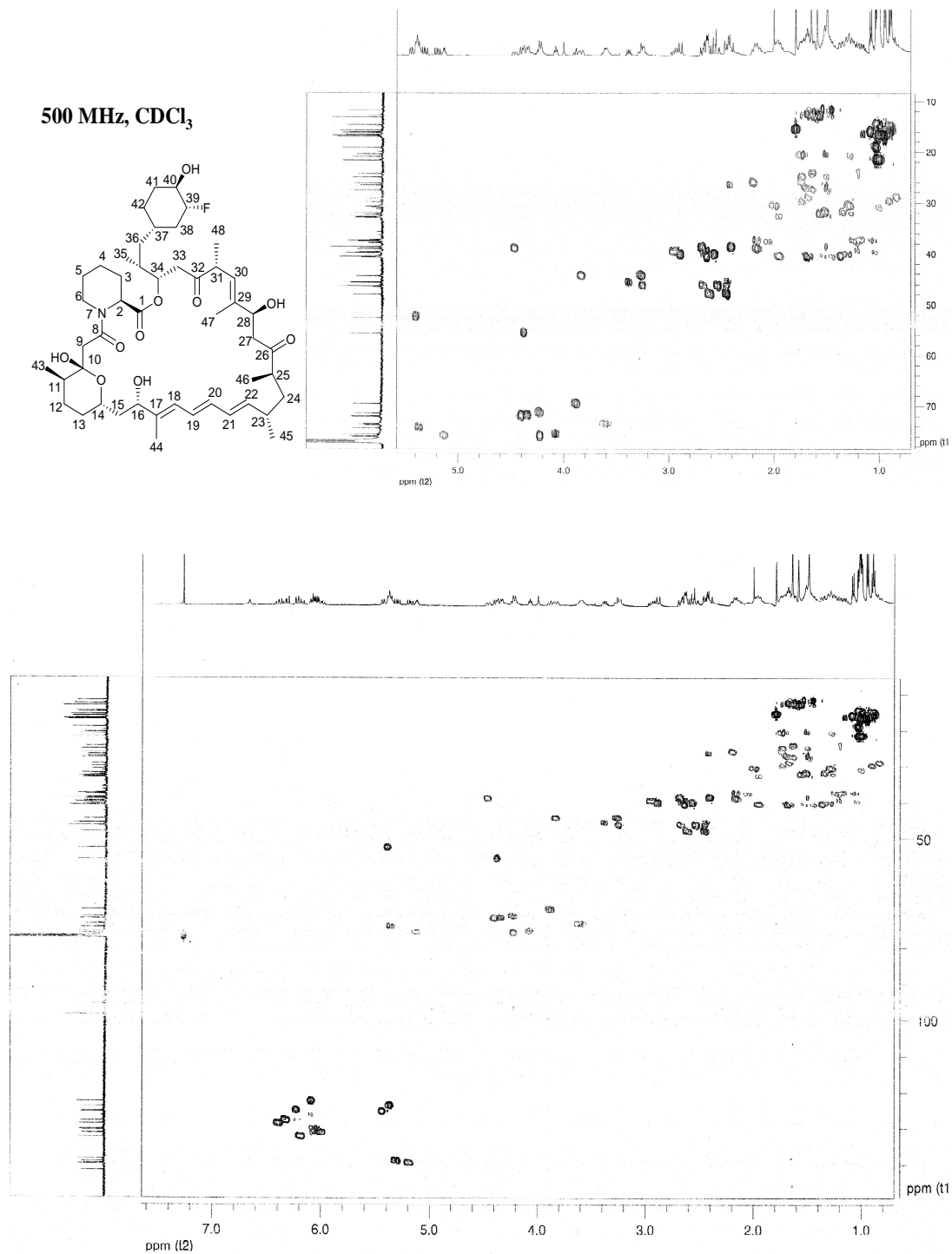
DEPT-90 NMR spectrum of fluoro-pre-rapamycin (**2.66**).

APPENDIX 4: COSY NMR Spectrum of Fluoroprerapamycin



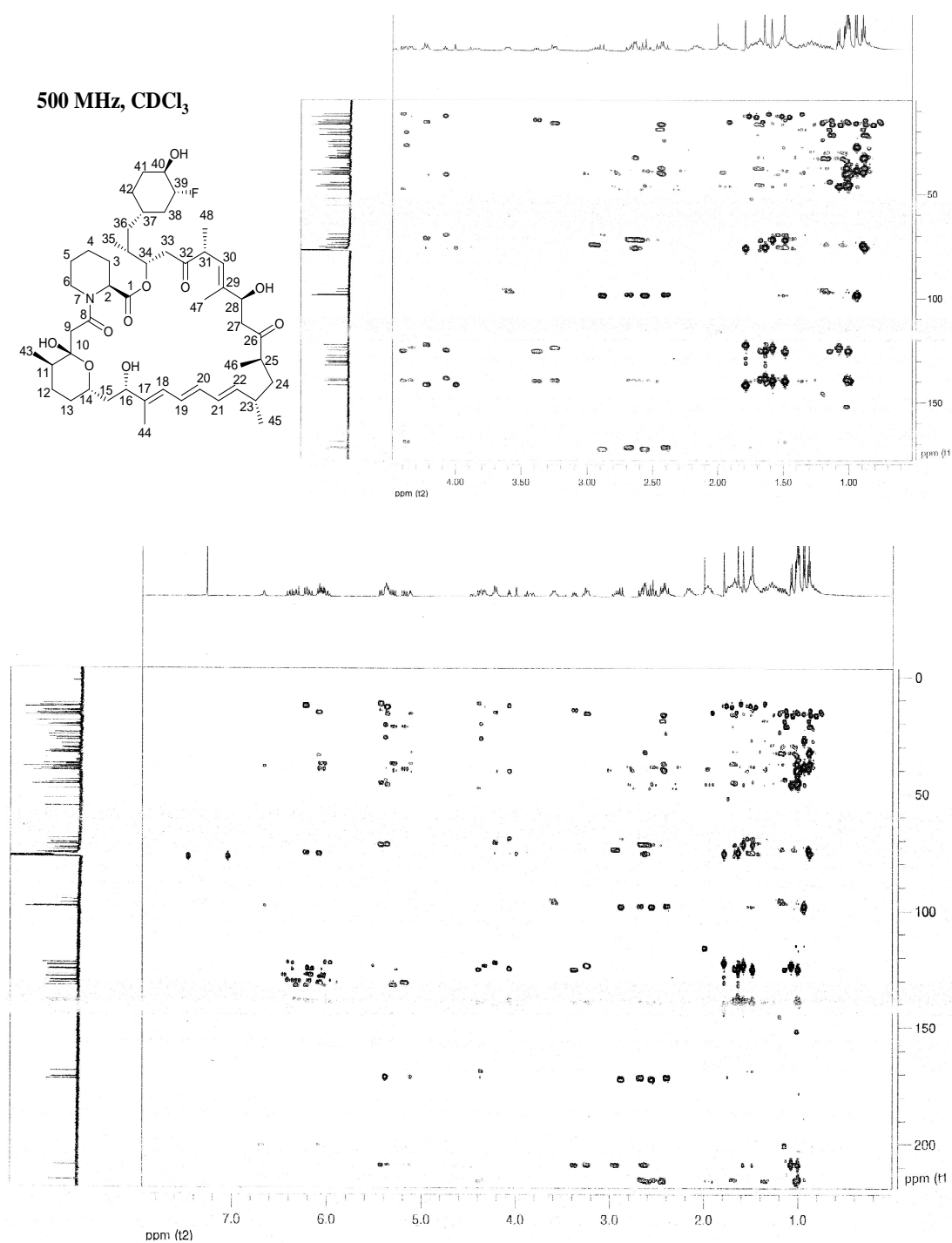
COSY NMR spectrum of fluoro-pre-rapamycin (2.66).

APPENDIX 5: HSQC NMR Spectrum of Fluoroprerapamycin



. HSQC NMR spectrum of fluoro-pre-rapamycin (2.66).

APPENDIX 6: HMBC NMR Spectrum of Fluoroprerapamycin



HMBC NMR spectrum of fluoro-pre-rapamycin (2.66).